AD_____

Award Number: DAMD17-97-1-7098

TITLE: Epitope Specific T Cell Immunity to Breast Cancer

PRINCIPAL INVESTIGATOR: Constantin G. Ioannides, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: June 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Final (1 Aug 97 - 31 May 02)

4. TITLE AND SUBTITLE
Epitope Specific T Cell Immunity to Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7098

6. AUTHOR(S)
Constantin G. Ioannides, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-Mail: cioannid@mdanderson.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Studies performed under this grant have made significant contributions to our understanding of the cellular immunity to the HER-2/neu protooncogene which is overexpressed ovarian cancer and a target for immunotherapy. These studies (1) identified novel epitopes recognized by $CD4^+$ cells from patients with both breast and ovarian cancer; (2) characterized the stimulatory ability for CTL induction of previously identified CTL epitopes in both in vitro and in vivo studies in the context of a clinical vaccine study; (3) demonstrated the Th1 inducing ability of the epitopes recognized by $CD4^+$ cells in the metastatic lymph nodes of patients with breast cancer; (4) performed a comparative analysis in metastatic and non-metastatic lymph nodes of the Ag-specific response to the CTL and helper epitopes. Most of the results of these studies have been published. In addition, a patent application based on the findings during this granting period has been submitted.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, CD4+ cells, in vivo studies	149
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The human HER-2/neu gene encodes a 185,000-kDa transmembrane tyrosine kinase growth factor receptor.(1) Overexpression of HER-2/neu occurs in up to one third of invasive breast cancers and is associated with aggressive disease, resistance to certain types of cytotoxic chemotherapy, and poor prognosis. (2-4) The HER-2/neu protein appears to be an excellent target for breast cancer vaccine development because it is expressed at very low levels in normal tissues but is overexpressed in aggressive primary invasive breast carcinomas and in breast cancer metastases.(7) The use of anti-HER-2/neu monoclonal antibodies has been shown in recent clinical trials to be effective in the treatment of women with HER-2/neu-overexpressing metastatic breast cancer.(8) Therefore, HER-2/neu vaccines might prove valuable in the development of immunity to breast cancer and in boosting the immune response in patients with established HER-2/neu-overexpressing tumors.

In breast cancer patients, the draining axillary LN are exposed to tumor-related cellular products and are often the first detectable site of metastases. Thus, the axillary LN represent an excellent and unique model in which to study the local immune response in breast cancer patients. Our laboratory has developed and tested several HER-2/neu peptides in an effort to better understand the mechanisms of tumor recognition by T lymphocytes and immunological tolerance to tumor, to characterize the structure of tumor antigens, and to potentially develop peptide-based vaccines. (9-11) We have previously shown that these HER-2/neu peptides may induce proliferative responses in peripheral blood T lymphocytes in some breast and ovarian cancer patients.(12) In the present investigation, we analyzed the differentiation, expansion, and cytokine secretion patterns from axillary LN lymphocytes stimulated with this HER-2/neu epitopes: the CTL epitope E75 and the helper epitope G89. The patients' lymphocytes were obtained from a HLA-$A2^+$ breast cancer patient with documented axillary LN metastases and a HER-2/neu overexpressing tumor. This study was designed to directly evaluate the local cellular immune response to HER-2/neu peptides in axillary LN from a woman with invasive carcinoma of the breast by characterizing the differentiation and cytokine responses of lymphocytes isolated from LN with metastases and from those without metastases.

Unexpectedly, we found that T lymphocytes from the LN without metastases (Met^-) expanded better at stimulation with CTL epitope E75 than T lymphocytes form the Met^+ LN. Addition of G89 to stimulation lead to a significant increase in nonpolarized "central memory" $E75^+$– TCR,

$CD45RO^+$ $CCR7^+$ cells in the Met^- but not in the Met^+ T-cells. Generation of Ag-specific "central memory" T-cells homing in lymphoid organs may provide a novel mechanism to control metastasis of HER-2^+ tumors.

BODY

Materials and Methods

Patient.

The patient had a stage IIIb, ER negative, PR negative, HER-2/neu positive left breast cancer. Biopsy of the left breast mass was read as metastatic invasive ductile carcinoma. She received induction chemotherapy with FAC. Left MRM and right total mastectomies were performed. Lymph nodes were collected under institutional (IRB) approved protocols.

Synthetic Peptides. The HER-2 peptides E75 (369-377) and G89(777-789) have been previously described ( ). They were prepared by the Synthetic Antigen Laboratory of M. D. Anderson Cancer Center and purified by HPLC. Peptides were dissolved in PBS and stored frozen at -20°C in aliquots until use.

Cells and Cell Lines. Freshly excised LN were collected in RPMI1640 medium with 10% FCS and antibiotics (complete RPMI medium), made single-cell suspension and cultured in the presence of low concentrations of IL-2 to induce division of live cells not affected by chemotherapy ($T^{IL\text{-}2}$ cells). This was considered necessary since a number of freshly isolated lymphocytes from LN died in the first days of culture, probably as a consequence of previous chemotherapy. Surviving $T^{IL\text{-}2}$ cells after 7-10 days cultures were used for experiments. HLA-A2 matched DC (dendritic cells) were isolated from PBMC of a healthy donor and cultured as described.

LN cells stimulation by G89. To generate "helper" cells, equal numbers of $T^{IL\text{-}2}$ cells from both Met^- and Met^+ LN were stimulated in parallel with G89 pulsed on autologous irradiated PBMC (G89-primed cells, G89-PR). Met^- and Met^+ LN cells co-cultured with irradiated PBMC in the absence of peptide were used as controls; no peptide primed cells (NP-PR). Four days later, IL-2 was added to all cultures, and maintained in cultures for 1 week. Afterwards, equal numbers of cells from all stimulation cultures Met^- LN - G89 - PR, Met^- LN - NP - PR, Met^+ - LN - G89 - PR, and Met^+ LN - NP - PR were irradiated at 20,000 Rad to stop their proliferation and added to

the cultures of Met^- LN T^{IL-2} (Met^- T^{IL-2}) and Met^+ LN T^{IL-2} cells. All cultures were stimulated with E75.

Stimulation of Met^- and Met^+ LN cells by E75. Each of Met^- T^{IL-2} cells and Met^+ T^{IL-2} cells were divided in two equal groups. Cells of both groups were stimulated with E75 either alone, or together with G89 (E75+ G89). LN cells stimulated with E75 received autologous irradiated NP-PR cells, while LN cells stimulated with E75 + G89 received autologous irradiated G89 - PR cells. For clarification, the stimulation procedure, the cell numbers, and the stimulation groups are shown in Table I. Separate stimulation controls were made using the same number of responding "helper cells" and amounts of peptides as in the groups 1-4. DC were not added to the control stimulation groups. Additional controls were made from Met^- T^{IL-2} and Met^+ T^{IL-2} cells, which were not stimulated with peptides but were cultured in parallel with changes in the medium and IL-2 as performed for the stimulation groups.

Flow Cytometry. The HLA-A2: Ig dimers (BD Pharmingen, San Diego, CA) were loaded with E75 (dE75) following the manufacturers instructions. Control dimers were prepared in the same conditions in parallel without addition of peptide (dNP). Cells were incubated with 20 µl of dE75 and dNP for 60 min and then were washed with PBS. dE75 and dNP bound on the cell surface were detected using goat-anti-mouse Ig G1 - PE (BD Pharmingen). Murine monoclonal anti-human CD45RO-FITC conjugated was used to detect surface expansion of CD45RO on the same cells. Murine monoclonal anti-human CCR7 followed by biotin conjugated rat-anti-mouse and streptavidin APC was used for detection of CCR7 on the same cells. All staining procedures from all samples were performed in the same experiment. All samples were analyzed in a FACS scan (BD) using the Cell Quest software in the same experiment.

Cytokine assays. Supernatants from all stimulation cultures were collected at the same time and analyzed for the presence and levels of IFN-γ, IL-10, IL-15, and IFN-α using ELISA (Bio Source International and R and D Systems) with a sensitivity of < 20pg/ml. IL-4 was determined using ultra-sensitive ELISA with a sensitivity of 4 pg/ml. (Bio Source).

Results

Stimulation of Met$^-$ T $^{IL\text{-}2}$ cells with E75 in the presence of G89 lead to a significant increase in E75$^+$ CD45RO$^+$ cells compared with Met$^+$ T $^{IL\text{-}2}$ cells.

To address whether stimulation with G89 in the presence of G89-PR cells affected expansion of E75 $^+$ TCR cells, both Met$^-$ $T^{IL\text{-}2}$ (Groups 1 and 2) and Met$^+$ $T^{IL\text{-}2}$ (Groups 3 and 4) cells were stimulated with E75 in the absence (Groups 1 and 3) or presence (Groups 2 and 4) of G89. NP-PR and G89-PR cells were irradiated before addition in the stimulation groups to ensure that their contribution is mainly due to Ag-MHC interaction and cytokines induced by stimulation with G89 (**Table I**). Seven days later, expression of E75 -TCR and of differentiation markers were determined by flow-cytometry. In separate experiments, we found that $T^{IL\text{-}2}$ cells from both Met$^-$ and Met$^+$ LN were $\geq$ 80% CD8$^+$ (data not shown). Analysis of the presence and number of E75-TCR$^+$ cells was performed in at least two independent experiments using various gate setting for negative controls (i.e., cells stained with dNP-PE conjugated, mouse FITC, and rat - IgG).

At least four independent determinations were made using various gate settings aimed at estimating the percentage of E75$^+$-TCR cells compared with controls-cells staining positive with dNP-PE conjugated. Gate settings which resulted in 0.05 - 0.1% FITC$^+$ - PE$^+$ or APC$^+$ - PE$^+$ cells in the negative control (dNP) also showed 30- to 40-fold increases in the number of E75-TCR$^+$ cells. Since such high calculated increases in E75-TCR$^+$ cells may be artefactual, we allowed gate settings in which cells stained with dNP-PE + anti-mouse FITC ranged between 0.1 - 0.4%. Using these settings, the initial numbers of E75-TCR$^+$ cells appeared higher, while the fold increase appeared lower than using settings with negative controls ranging between 0.01-0.05. E75 $^+$ CD45RO $^+$ cells significantly increased in number during 7 days of culture. E75$^+$ - TCR cells increased in the Met$^-$$T^{IL\text{-}2}$ from 4.46 % in the control cells grown in IL-2 to 19.46 % and 20.79% in the Groups 1 and 2, respectively. In contrast, the increase in E75$^+$-TCR cells from Met$^+$ $T^{IL\text{-}2}$ was approximately half of what was obtained for the Met$^-$ $T^{IL\text{-}2}$ cells: from 4.46% in the presence of IL-2 to 11.24% and 12.94% in Groups 3 and 4, respectively.

When the numbers of $E75^+$ $CD45RO^+$ cells were calculated based on the number of live, recovered cells in the cultures, we found that Group 1, $E75^+$ $CD45RO^+$ cells expanded by 8.4-fold, while Group 2, $E75^+$ $CD45RO^+$ cells expanded by 11.5-fold compared with cells which were not stimulated. Group 3, $E75^+$ $CD45RO^+$ cells from Met^+ LN increased in numbers only by 6-fold compared with control unstimulated T^{IL-2} cells. The differences were significantly higher between Groups 2 and 4. $E75^+$ $CD45RO^+$ cells increased only by 6-fold in Group 4 compared with Group 2. This suggested that although the initial numbers of $E75^+$ cells in Met^- LN and Met^+ LN were similar, T^{IL-2} cells from Met^- LN had a better ability to expand at stimulation with E75.

Stimulation with E75 induced T-cell proliferation and differentiation to central memory (CM) T-cells. The central memory T-cells are characterized by the presence of the CCR7 on their surface. To address how E75 + G89 stimulation affected expression of this differentiation marker, both Met^- and Met^+ T^{IL-2} cells were analyzed for the presence of $E75^+$ $CD45RO^+$ $CCR7^+$ cells. Again there was only a small (20%) increase in $E75^+$ $CCR7^-$ cells in Met^+ T^{IL-2} cells, but there was no increase in $E75^+$ $CCR7^-$ cells in Met^+ LN.

These results suggested that E75 + G89 induced an increase in central memory (CM) cells. Results from calculations of cell numbers show that (Figure 1) $E75^+$ $CD45RO^+$ $CCR7^+$ cells increased in number by 13.2-fold in the Group 2 compared with 8.4-fold in Group 1. The increase in $E75^+$ $CCR7^+$ cells accounted for almost of the entire increase in $E75^+$ $CD45RO^+$ cells in Group 2 comparing with Group 1. This response was not observed with Met^+ T^{IL-2} cells. Group 4 $E75^+$ CD45 RO^+ $CCR7^+$ cells were not higher in number than the corresponding population in Group 3. $E75^+$ -TCR CM cells preferentially increased at stimulation with G89 but only in the Met^- T^{IL-2} cells.

The numbers of $E75^+$ TCR $CD45RO^+$ $CCR7^+$ obtained after stimulation with E75 were again two-fold higher in Met^- T^{IL-2} cells than in Met^+ T^{IL-2} cells. However, E75 + G89 had a significantly weaker effect in expansion of these cells from Met^- T^{IL-2} cells and no effect in Met^+ T^{IL-2} cells. (Figure 1 A, B column 3). Overall, these results show that stimulations with E75 significantly increased the number of $CD45RO^+$ cells, while addition of G89 and of G89-PR

cells not only lead to further expansion of $E75^+$ - TCR, $CD45RO^+$ cells but preferentially increased the $CCR7^+$ $CD45R0^+$ subset. Expansion of the $CCR7^+$ subset in response to G89 was restricted to the Met^- T^{IL-2} cells.

Cytokine induction in Met $^-$ LN and Met $^+$ LN at stimulation with E75 and G89. To address whether the differences in the number of CM cells in Met^- and Met^+ populations, and in the $E75^-$ stimulated versus E75 + G89 stimulated cells were related to qualitative or quantitative differences in induction of one cytokine or combinations of cytokines by stimulation with E75 and G89, we determined the levels of expression of IFN-γ as representative Th1 cytokine, IL-4 as representative Th-2 cytokine and of IL-10, as suppressor cytokine. The levels of IL-15 and of IFN-α (as survival cytokines) for memory cells were also determined. Figure 2A shows that during the first 24 hours of stimulation, Group 2 cells secreted more IL-15 than Group 1 cells. This was paralleled by induction of IFN-α. IFN-α plus activated $CD4^+$ are required to induce rapid secretion of IL-15. The levels of IL-15 increased and reached similar levels in both groups on day three. The levels of IL-15 were significantly higher in Groups 1 and 2 than in Group 4 . The results in Figure 2 show that the levels of IFN-γ induced by stimulation with E75 (Group 1) and E75 + G89 (Group 2) were similar over the first six days in culture. The levels of IFN-γ in Group 2 were significantly lower than the levels of IFN-γ in Groups 3 and 4 although the difference was less than two fold. The levels of IL-4 were similar in all four stimulation groups over the five-day period. It is interesting to note that the levels of IL-4, decreased in all stimulation groups over the five-day period. Thus, except for day one, stimulation with E75 and G89 did not result in a significant change in the IFN-γ: IL-4 ratios between MET^- T cells and Met^+ T cells. In contrast with IL-4, the levels of IL-10 were higher by several fold in Groups 3 and 4. The level of IL-10 were low on day one in Groups 1 and 2, but increased during the following days. Thus, determination of the levels and kinetics of secretion of Th1 and Th2 cytokines did not show any significant differences between Groups 1 and 2.

These results indicate that stimulation with E75 + G89 lead to higher initial levels of IFN-α and IL-15 in the Met $^-$ T^{IL-2} cells, but not in the Met^+ T^{IL-2} cells. In contrast, both E75 an dE75+G89 induced higher levels of IL-10 in Met^+ T^{IL-2} cells than in Met^- cells. This raised the possibility

that the observed increase in $E75^+$ - TCR CM cells was due to activation of DC by $G89^+$ cells present in Met^- LN.

Discussion

In this report, we analyzed the differentiation, expansion, and cytokine secretion by T lymphocytes from Met^- and Met^+ LN cells of a breast cancer patient in response to epitopes from HER-2/neu recognized by $CD8^+$ and $CD4^+$ cells. The unique availability of T-cells from both metastatic and non-metastatic LN allowed comparisons in the responses of the cells from the same individual in experiments performed in the same time.

Unexpectedly, we found that stimulation with the peptide mapping the CTL epitope E75 induced significant expansion, by more than 8-fold, of $E75^+$ - TCR $CD45RO^+$ cells and by 13 fold of $E75^+$ -TCR $CD45RO^+$ $CCR7^+$ cells from the Met^-LN, but only by two-fold of the corresponding cells from the Met^+ LN. G89 had a less significantly effect in expansion of $CCR7^+$ cells from Met^+ LN. E75 had a much stronger effect (eight fold increase) in expansion of $CCR7^-$ cells in Met^- LN than of $CCR7^-$ cells of Met^+ LN (three fold increase). In the same experiment, the effects of $E75^+$ G89 in expansion of $CCR7^-$ were similar with that of E75 alone, indicating that the effect of G89 were diverted mainly to the $CCR7^+$ subset. Since the initial numbers of $E75^+$ $CD45RO^+$ and $E75^+$ $CD45RO^+$ $CCR7^+$ cells were similar in the $T^{IL\text{-}2}$ cells, the same DC were used as APC, and the same NP-PR and G89-PR cells were used as "helpers", these results indicate that $E75^+$ cells in the Met^- LN and Met^+ LN were endowed with distinct capacity for proliferation and differentiation in response to E75 + G89. This conclusion is also supported by the fact that cells from both LN were cultured in medium containing IL-2 for at least one week before stimulation, and the starting numbers of $E75^+$ cells in both $T^{IL\text{-}2}$ samples were similar.

A novel finding of this study was that $E75^+$ TCR $CD45RO^+$ cells and $E75^+$- TCR $CD45RO^+$ $CCR7^+$ cells from the Met^- LN expanded better in the presence of E75 + G89 compared with E75 alone. In fact, the entire increase in cell numbers in Group 2 compared with Group 1 was due to the increase in $E75^+$ $CD45RO^+$ $CCR7^+$ cells. This suggests that G89 had preferential effects on $CCR7^+$ cells.

The levels of Th1 and Th1 cytokines in the culture environment did not indicate any significant association between the phenotype and the levels of a particular cytokine. Groups 1 and 2 cells from Met^- T^{IL-2} cells responded with significantly lower initial levels of IL-10 than Met^+ T^{IL-2} cells, and higher IFN-γ: IL-10 ratios than Groups 3 and 4. The differences in IFN-γ and IL-10 levels between the Groups 1 and 2 were small. The high release of IL-10 from cells of Groups 3 and 4 may account for the differences in proliferation between the Met^- LN and Met^+ LN cells.

Group 2 cells secreted higher levels of IFN-α at stimulation with $E75^+$ G89 than at stimulation with E75 alone. This was paralleled by high and rapid induction of IL-15 in the APC. Since rapid induction of IL-15 in the DC requires IFN-γ and co-stimulation from $CD4^+$ cells, more likely though the involvement of CD40L, this raised the possibility that G89 presented by DC enhanced expression of CD40L or of another receptor involved in IFN-α induction at high levels. This may be supported by the fact that Group 1 contained NP-PR cells as helpers, while Group 2 contained G89-PR cells as helpers. Groups 1 and 2 differed by the levels and kinetics of induction of the IL-15 and IFN-α. Both IFN-α and IL-15 have been described to be involved in survival and expansion of $CD8^+$ memory cells.

Recent evidence indicates that memory $CD4^+$ and $CD8^+$ T-cells contain at least two functional subsets: (a) "central memory" (CM) nonpolarized which express the chemokine receptor CCR7 and home to lymph nodes, and (b) "effector memory" which have lost the expression of CCR7 and migrate to the non-lymphoid organs. Since our $CCR7^-$ cells were obtained from LN, and were expanded with IL-2 before stimulation, it is more likely that they were not immigrants from inflamed tissues but rather generated in-vitro after stimulation with Ag and expansion in IL-2.

The reasons for which $CCR7^+$ cells of CM phenotype increased at stimulation with E75 and E75 + G89 in the Met^- LN but not in Met^+ T-cells are unclear. Irreversible differences may be induced by the tumor invasion, which cannot be overcome by culture in medium with IL-2. If expansion of $CCR7^+$ with maintenance of CM phenotype required the presence of $G89^+$ cells, it is possible that such a population was absent or significantly diminished in the Met^+ LN. The presence of "help" fromG89-PR cells was not sufficient to expand $CCR7^+$ cells or to induce IFN-

α from the Met^+ LN cells, or IL-15 from DC. Because of lack of G89-dimers, the population of G89 TCR^+ cells cannot be identified at this time. It is tempting to speculate that such $G89^+$ regulatory helper cells are present in the lymph nodes and they are eliminated by the tumor or epithelium-induced factors. Further studies are needed to address this point. A third possibility is that a tumor secreted factor affects/inhibits production of a soluble factor needed for maintenance of the undifferentiated CM state or of cells producing this soluble factor.

Another possibility is that survival and expansion of $CCR7^+$ depended on a factor/cytokine distanct from IL-15 which is produced by DC upon encounter with $G89^+$ T-cells. The reasons for the differences in the magnitude of the response to E75 and E75 + G89 of Met^- and Met^+ cells deserve further investigation.

KEY RESEARCH ACCOMPLISHMENTS

1. Identification of HER-2/neu T cell epitopes which induce proliferation and cytokines from $CD4^+$ cells of ovarian and breast cancer patients (Publications No. 1 and 2).

2. Identification of protection of lysis of tumor by the NK cells, and inhibition of NK cytotoxicity by the epitopes C85, E89, and E75 which are recognized by CTL (Publication No. 3).

3. Identification of a pattern of Th1 cytokine responses in axillary lymph nodes induced by helper peptides in patients with carcinoma of the breast (Publication No. 4).

4. Identification of determinant spreading and characterization of the direction of spreading from one epitope to another (Publication No. 5).

5. Identification of approaches to increase CTL epitope presentation by the tumor through induction of accelerated HER-2 degradation using (1) geldanamycin (GA) and (2) phosphorylation agonists (EGF, NDF) together with phosphatase inhibitors (sodium vanadate) (Publications No. 6 & 7). A part of these studies was supported by another grant.

6. Characterization of immunogenicity of CTL epitopes and of the nature of "help' provided by the "helper epitopes": (1) HER-2 CTL epitope E75 is weak and partial agonist; (2) Stimulation with such agonists induce frequently IFN-γ from responders, but less frequently cytolytic function; (3) helper peptide (G89) activated $CD4^+$ cells regulate the survival of Ag-specific CTL. This regulation has opposite effects depending on the application of the helper peptide. In the induction phase, G89 enhances expansion and Ag-dependent cytolysis at priming with a CTL epitope E75. At restimulation, G89 inhibited the survival of the E75-specific CTL. These effects were paralleled by changes in the IFN-γ: IL-10 balance. Higher levels of IFN-γ at restimulation were associated with death rather than expansion. (4) the helper effect of G89 was characterized by comparative analysis of responses in a metastatic LN versus a non-metastatic LN. Although G89 helped expansion of $E75^+$ cells, the responses were much higher in the non-metastatic than in the metastatic LN ((Publications No. 8,9,10 and this report).

7. An unexpected research opportunity resulted from the development of computer-based modeling of the peptide HLA-A2 complexes, without requirement for isolation and crystallographic analysis. This opportunity translated in our ability to develop C-side chain variants of the epitope. Using such C-side chain variants, we discovered a novel approach to enhance survival and prevent apoptosis in tumor Ag activated CTL. This approach consists in CTL priming and expansion using a weaker agonist than the nominal Ag. This assures that responsders will not die by overstimulation or unbalanced stimulation. Then, once the responders increase their levels of Bcl-2, and respond to Ag + αFas by proliferation, activation by the wild-type Ag can further expand the effectors to large numbers (Publication No. 11, in press).

8. Immunological monitoring for effector function of lymphocytes from breast cancer patients vaccinated with E75 was also supported by this grant because such studies were both significant and relevant for breast cancer patients (Publication No.12, in press).

REPORTABLE OUTCOMES

1. Identification of T cell epitopes which induce proliferation, cytokine secretion, determinant spreading and provide help for CTL induction.

2. Characterization of the help revealed that such helper epitopes have potentating effects on CTL expansion and activation of their cytotoxic function when used at priming. However, their use at restimulation (boosting) amplifies the apoptosis inducing effects of the CTL epitope plus IL-2.

3. Identification of tumor Ag variants which can modulate the survival of CTL.

4. Identification of the ability of the HER-2 CTL epitope to induce immune responses in patients with breast cancer in a vaccine study.

CONCLUSIONS

As indicated by the attached publications and the final report, studies supported by Grant 1-7098 concluded that:

1. Epitopes which can activate $CD4^+$ cells are present in HER-2/neu molecules. Such epitopes can induce a Th1 response which may be useful in the induction phase of a CTL response, but not in later phases. In fact, Th1 help accelerate responder decay if used later. This is significant for design of vaccines containing helper plus cytolytic epitopes.

2. The helper epitopes are functional for activation of metastatic LN. This helper effect is characterized by an increase in the number of Ag-specific T cells. This can be beneficial for vaccination of patients with LN metastases. An additional important point is that based on the much higher response observed is that vaccination may be more effective if used earlier in patients without LN involvement, with primary disease, and in patients without evidence of disease.

3. Studies on the effect of GA, EGF, and NDF in tumor Ag presentation lead to an interesting an novel conclusion, i.e., CTL epitopes presented by the tumor derive from nascent proteins of short half-life, and at much lesser extent from the large amount of transmembrane protein. Generation of such fast degrading Ag precursor holds promise for design and development of genetic vaccination.

REFERENCE:

1. Fisk, B., Hudson, J. M., Kavanagh, J., Murray, J. L., Wharton, J. T., **Ioannides, C. G.,** and Kudelka, A. P. Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patient to HER-2 peptides. *Anticancer Res.* 17:169-179, 1997.

2. Tuttle, T.M., Anderson, B.W., Thompson, W.E., Lee, J.E., Sahin, A., Smith, T.L., Grabstein, K.H., Wharton,J.T., **Ioannides, C.G.,** Murray, J.L. Proliferative and Cytokine responses to class II HER-2/neu-associated peptides in breast cancer patients. *Clin. Cancer Res., 4:2015-2024, 1998.*

3. Anderson, L. D. Jr., Hudson, J. M., Savary, C. A., Fisk, B, Gershenson, D. M., and **Ioannides, C.G.** HER-2/neu peptide specificity in the recognition of HLA-A2 by natural killer cells. *Cancer Immunol Immunother,* 48(7):401-410, 1999.

4. Kuerer, H., Peoples, A., Sahin, J., Murray, J., Singletary, E., Castilleja, A., Hunt, K., Gershenson, D., and **Ioannides. C.G.** Axillary lymph node (LN) cellular immune response to HER-2/neu peptides in patients with carcinoma of the breast. *J. Interferon and Cytokine Res., 22:583-592, 2002.*

5. Anderson, B.W., Kudelka, A.P., Honda, T., Pollack, M.S., Gershenson, D.M., Gillogly, M.A., Murray, J.L. and **Ioannides, C.G.** Induction of determinant spreading and Th1 responses by in vitro stimulation with HER-2 peptides. *Cancer Immunology Immunotherapy 49:459-468, 2000.*

6. Castilleja, A., Ward, N.E., Epstein, R.B., Kudelka, A.P., Efferson, C.L., Gershenson, D.M., O'Brian, C.A. and **Ioannides, C.G.** Treatment with HER-2 Phosphorylation agonists enhance tumor ability to stimulate epitope specific CTL *in vitro. Oncology Reports: 9:929-935, 2002.*

7. Castilleja, A., Ward, N.E., O'Brian, C.A., Swearingen II, B., Swan, E., Gillogly, M.A., Murray, J.L., Kudelka, A.P., Gershenson, D.M. and **Ioannides, C.G.** Accelerated HER-2 degradation enhances ovarian tumor recognition by CTL. Implications for tumor immunogenicity. *Molec. Cell. Biochem. 217:21-33, 2001*

8. Lee, T.V., Johnston, D.A., Thomakos, N., Honda, T., Efferson, C.L., and **Ioannides, C.G.** Helper peptide G89 (HER-2:777-789) and G89-activated cells regulate the survival of effectors induced by the CTL epitope E75 (HER-2, 369-377) Correlation with the IFN-γ: IL-10 balance. *Anticancer Res., 22(3):1481-1490, 2002.*

9. Lee, T. V., Anderson, B. W., Peoples, G.E., Castilleja, A., Murray, J.L., Gershenson, D. M. and **Ioannides, C.G.** Identification of activated tumor-Ag-reactive $CD8^+$ cells in healthy individuals, *Oncology Reports, 7:455-466, 2000.*

10. Anderson, B.W., Peoples, G.E., Murray, J.L., Gillogly, M.A., Gershenson, D.M., and **Ioannides, C.G.** Peptide priming of cytolytic activity to HER-2 epitope 369-377 in healthy individuals. *Clin. Cancer Res. 6:4192-4200, 2000.*

11. Castilleja, A., Carter, D., Efferson, C., Ward, E., Fisk, B., Kudelka, A.P., Gershenson, DM, Murray JL, O'Brian, C.A., and **Ioannides, C.G.** Induction of tumor-reactive CTL by C-side chain variants of the CTL epitope HER-2 (369-377) selected by molecular modeling of the peptide: HLA-A2. *J. Immunology, 169:00-00,2002. In Press.*

12. Murray, J.L., Gillogly, M., Przepiorka, D., Brewer, H., Ibrahim, N., Booser, D., Hortobagy, G.N., Kudelka, A.P., Grabstein, K., Cheever, M. and **Ioannides, C.G.** Toxicity and induction E75-specific tumor lytic CTL by HER2Peptide E75-369-377) combined with GM-CSF in HLA-$A2^+$ patients with metastatic breast and ovarian cancer: A Phase I vaccine trial. *Clin. Cancer Res., 8:-00-00, 2002.*

Table I

	Stimulation Groups			
	Met^- LN		Met^+ LN	
APC:	1	2	3	4
	DC	DC	DC	DC
	E75	E75	E75	E75
	0	G89	0	G89
	NP-PR	G89-PR	NP-PR	G89-PR
Responders:	Met^- LN	Met^- LN	Met^+ LN	Met^+ LN
Recovered Cells Total:	2.90×10^6	3.70×10^6	3.44×10^6	2.90×10^6

E75 and G89 were added at 30 μg/ml. DC were added at 1.5×10^5/well, while responders at 1.5×10^6/well (S:R ratio of 1:10). Irradiated NP-PR and G89-PR cells were added at 3.0×10^5/well. "Helper" - APC ratio of 2:1. "Helper" - Responder ratio of 1:3. NP-PR and G89-PR cells were irradiated (20,000 Rad) before addition to the cultures. In Groups 1 and 2, NP-PR and G89-PR cells were from nonmetastatic (Met^-) LN, while in Groups 3 and 4, NP-PR and G89-PR cells were from metastatic Met^+ LN. Met^- LN and Met^+ LN indicate that responders were from non metastatic and metastatic LN respectively.

Legends To The Figures

Figure 1. Total numbers of $E75^+$ $CD45RO^+$ $CCR7^-$ (incomplete differentiated effectors) and $E75^+$ $CD45RO^+$ $CCR7^+$ (CM cells) in the Met^- LN (A) and the Met^+ LN (B). Results of one of two determinations are shown.

Figure 2. Induction of IL-15 (A), IL-4 (B), IL-10 (C), and IFN-γ (D) at stimulation with E75 and E75 + G89. Groups 1 and 2 (Met^- LN), Groups 3 and 4 (Met^+ LN)

Figure 1

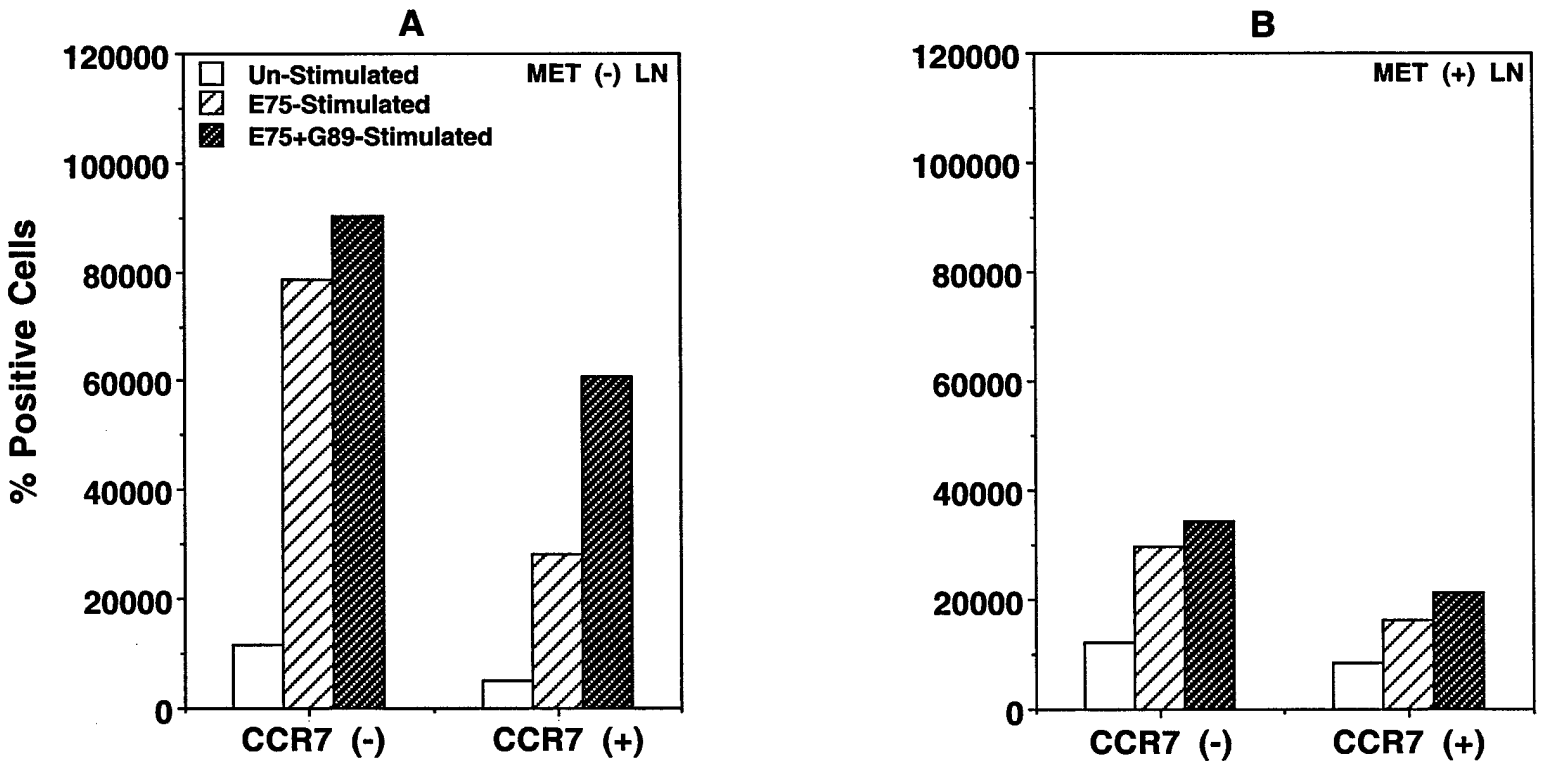
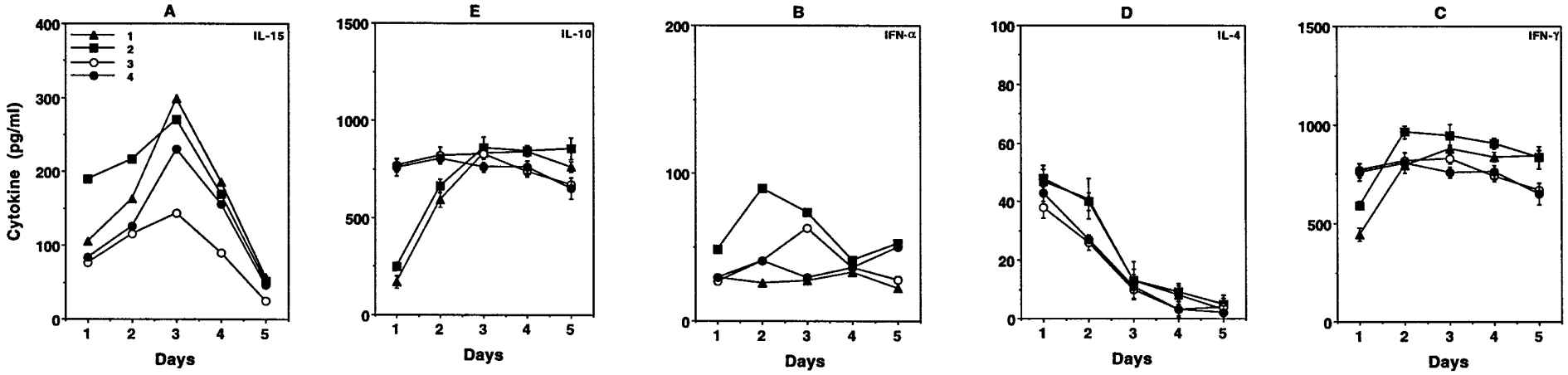

A
B
% Positive Cells
120000
100000
80000
60000
40000
20000
0
Un-Stimulated
E75-Stimulated
E75+G89-Stimulated
MET (-) LN
MET (+) LN
CCR7 (-)
CCR7 (+)

Figure 2


A
IL-15
1
2
3
4
Cytokine (pg/ml)
400
300
200
100
0
1 2 3 4 5
Days
E
IL-10
1500
1000
500
0
1 2 3 4 5
Days
B
IFN-α
200
100
0
1 2 3 4 5
Days
D
IL-4
100
80
60
40
20
0
1 2 3 4 5
Days
C
IFN-γ
1500
1000
500
0
1 2 3 4 5
Days

PERSONNEL SUPPORTED BY THE GRANT

1. Constantin G. Ioannides, Ph.D.
2. Clay Efferson, M.Sc.
3. Byung K. Ko, M.D., Ph.D.
4. Scott A. Kelly, B.Sc.
5. Michael Gillogly, B.Sc.
6. Tom V. Lee, B.Sc.
7. Sara Mouzi

APPENDICES

ANTICANCER RESEARCH *17*: 45-54 (1997)

Existent Proliferative Responses of Peripheral Blood Mononuclear Cells from Healthy Donors and Ovarian Cancer Patients to HER-2 Peptides*

BRYAN FISK[1], J. MICHAEL HUDSON[1], JOHN KAVANAGH[2], J. TAYLOR WHARTON[2], JAMES LEE MURRAY[3], CONSTANTIN G. IOANNIDES[1] and ANDRZEJ P. KUDELKA[2]

The Departments of [1]Gynecologic Oncology, [2]Clinical Investigations, Section of Gynecologic Medical Oncology, and 3Bioimmunotherapy, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, U.S.A.

Abstract. *Identifying target antigens for tumor-reactive T cells is important for understanding the mechanisms of tumor escape and developing novel anticancer therapies. To date, mainly CTL responses from tumor infiltrating/ associated lymphocytes (TIL/TAL) to peptide antigens have been investigated in ovarian cancer. In the present study, the ability of self-peptides derived from HER-2/neu proto-oncogene product (HER-2) to stimulate proliferation of PBMC from healthy donors and ovarian cancer patients has been assessed. Peptide sequences from HER-2 containing anchors for major human MHC-class II molecules have been identified. These peptides induced proliferative and cytokine responses at higher frequency in healthy donors than ovarian cancer patients. Four HER-2 peptides corresponding to positions: 396 - 406, 474 - 487, 777 - 789, and 884 - 899 were able to stimulate proliferation of a larger number of healthy donors than three other distinct HER-2 peptides 449 - 464, 975 - 987 and 1086 - 1098. The pattern of responses of twenty five ovarian cancer patients was different from that in healthy donors. T cell lines were developed by stimulation with peptides from PBMC of an ovarian cancer patient who showed a stable response to all four HER-2 peptides for over six months. Each T cell line was different in its ability to secrete IFN-γ and IL-10. These results demonstrate (a) that self-peptides from HER-2 can stimulate expansion of T cells in both healthy donors and ovarian cancer patients, and (b) the ability of different peptides to stimulate secretion of different cytokines from lymphocytes of ovarian cancer patients. These results may be important for understanding the mechanisms of tolerance and autoimmunity in human cancers.*

The HER-2/neu proto-oncogene product (HER-2) is the target of autoantibodies in breast cancer (1) and of cytotoxic T lymphocytes (CTL) in ovarian, breast, and lung cancer (2-5). Since these auto-antibodies are specific for the native conformation of the HER-2, they must be induced by the native molecule. HER-2 is present in both healthy individuals and cancer patients. Similarly HER-2-reactive CTL are specific for a number of epitopes (2-5) of which one HER-2, 369-377 was found to be immunodominant in our studies (5). These CTL were isolated from lymphocytes associated with ovarian tumors in patients with advanced disease after culture in the presence of IL-2. This suggests that $CD4^+$ T cells capable of helping either B cells, or $CD8^+$ CTL, or both may be present in the cancer patients. This raises the question as to whether CD4+ T cells capable of recognizing epitopes from human HER-2 are also present. Previous studies have

Abbreviations: Cytotoxic T Lymphocytes, CTL, Position, P; T cell receptor, TCR; HER-2/neu proto-oncogene, HER-2; stimulation index, S.I.; standard deviation, SD, TT, tetanous toxoid.

*Acknowledgments. We thank Ms. Rosario Mante for collecting specimens and Ms. Susan Mondragon for the outstanding preparation of this manuscript. Peptide synthesis was supported in part by core grant 16672.

Correspondence to: Dr. Constantin G. Ioannides, Department of Gynecologic Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 67, Houston, Texas 77030, USA.

Key Words: HER-2, $CD4^+$, epitope, ovarian cancer, Th1, Th2, cytokines.

0250-7005/97 $2.00+.40

Table I. *Sequences of HER-2 peptides.*

PeptideCode	Position	Sequence[a]
F11	5- 19	A L C R W G L L L A L L P P G
D122	396 - 406	Q L Q V F E T L E E T
F12	449 - 464	G I S *W* L G **L** R S **R** E **L** G S G L
F14	474 - 487	T V P *W* D Q *L* F *R* N P H Q A
F7	776 - 789	G S P *Y* V S **R** L **L** G I C L
F6	777 - 797	G S P *Y* V S **R** L *L* G I C L T S T V Q
F8	832 - 851	G M S *Y* L E D V R L V H R D L A A R N
F10	975 - 997	F S R *M* A R S P Q R *F* V V **I** Q N **E** D **L** G
D100	1086-1098	F D G D L M G A A K G L
F13	884 - 899	V P I K *W* **M** A L **E** S **I** L R R R F

[a]Potential DR4 anchors that distinguish between DRB1*0401/0404 and DRB 1*0402 are shown in bold. Tyr (Y) and Trp (W) residues characteristic of the P1 anchors for DRB1*0401 and DRB*0101 binding motifs (21) are italicized. Prolines for protection from proteolysis are underlined. Peptide F10 extends a potential helper epitope after the CTL epitope C85 (the sequence is underlined). Both DR4 allotypes (DR4.1 and DR4.2) accept peptides with Leu, Ile, Met, Phe, and Val as P1 anchor residues (19).

shown that $CD4^+$ T cells with specificity for HER-2 can be identified in breast cancer patients (1). The extent and the existence of autoreactive T cell repertoire to HER-2 in both healthy humans and ovarian cancer patients has not been previously identified. To assess the speciflcity of these T cells and identify potential targets for epitope-specific immunotherapy, we investigated the responses to HER-2 of a group of patients with ovarian cancer subsequent to chemotherapy and a group of healthy individuals.

To characterize the T cell response to HER-2 we assessed a number of T cell epitopes of HER-2 with a set of synthetic peptides based on the HER-2 sequence. We wanted to identify a set of such peptides to which healthy donors and ovarian cancer patients respond by proliferation and determine the frequency of these responses. We stimulated PBMC from twenty five ovarian cancer patients and fourteen healthy donors with synthetic HER-2 peptides. PBMC from each donor were stimulated individually with each peptide, but not with pooled peptides. The general pattem of response was characterized by a group of four HER-2 peptides designated as D122:HER-2,396 - 406, F7:HER-2:777 - 789 F13:HER-2,884 - 899, F14:HER-2, 474 - 487 which induced a signiflcantly higher frequency of responses than the other three HER-2 peptides designated as F10:HER-2,975 - 997, F12:449 - 464, D100:HER-2,1086 - 1098 in both healthy donors and ovarian cancer patients. The frequency of responses to most HER-2 peptides was significantly lower in ovarian cancer patients who had received chemotherapy than in healthy donors.

T cell lines were raised against individual HER-2 epitopes represented by peptides F7, F13, F14 and D122 from PBMC of an ovarian cancer patient by restimulation with HER-2 peptides and expansion in IL2. Thes̨ T cell lines showed a different pattern of IFN-γ and IL-10 production. F13 induced T cells secreted significantly higher amounts of IFN-γ than IL-10 while F7 and F 14 induced T cells secreted significantly higher levels of IL-10 than F13 and D122 induced cells.

Materials and Methods

Cells. Peripheral blood nononuclear cells (PBMC) were obtained from fourteen healthy donors and twenty flve ovarian cancer patients. All patients had advanced disease. After initial surgery, they were treated with platinum (cisplatin or carboplatin). One patient was receiving primary platinum based chemotherapy. The other twenty four patients had received additional chemotherapy. The latter was either carboplatin reinduction, salvage therapy with paclitaxel or experimental therapy with several different drugs. Blood collection was made at least three weeks after the last chemotherapy administration. PBMC were isolated from heparinized peripheral blood as described (1,6). At the time of the assay the patients were not receiving chemotherapy.

HER-2 epitopes selection. Peptides to be tested in the proliferation assays were selected based on the T cell sites in HER-2 predicted by the

computer program ANT.FIND.M, the general binding motif for human class MHC-II antigens, and the presence in the sequence of anchors for a number of MHC-class II antigens: HLA-DRl, HLA-DR3, HLA-DR4, HLA-DRll, and HLA-DQ7 (7-13). The sum of the allelic frequencies of these antigens cover approximately 100% of the Caucasian and Hispanic populations and between 75 - 92% of the African American and Asian populations. For example HLAD97 is present at 28% (Caucasians) 23% (African Americans), and at 43% (Hispanics). Similarly, each of HLA-DR1, DR3, DR4 and DR11 is present between 17 - 20% in each of the major population groups. The general binding motif for various human MHC-class II molecules consist of a position 1 (P1) anchor, *i.e.* an aromatic or large aliphatic residue in the first 3 - 5 amino acids close to the N-terminus, and other major but less essential anchors at positions 4, 5 - 7, and 9 counting from the Pl anchor (13). A large number of "promiscuous" peptides are capable of binding to many different MHC-class II molecules (13, 14), because their sequences contain overlapping binding motifs for MHC-class II molecules (13, 14). The search for specific anchors for these major MHC-class II antigens in the HER-2 sequence indicated significantly more candidate epitopes for binding to HLA-DR1 (n = >20) than for HLA-DR3 (n = 11), than for binding to HLA-DR4, HLA-DQ7, and HLA-DR11.

Ten HER-2 peptides 11 - 22 residues long (Table I) were synthesized by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, using a solid phase method as previously described (2). The identity of peptides was determined by amino acid analysis. The purity of peptides ranged between 93 - 97% as determined by HPLC. Peptides were dissolved in PBS, aliquoted at 2 mg/ml and stored frozen at -20°C until use. The codes used to identify these HER-2 peptides in this paper were assigned by the Synthetic Ag Laboratory. To ensure better representation of different binding motifs for these MHC class II antigens, at least two peptides were synthesized containing anchors for each of HLA-DR1, -DR3, and -DQ7. When possible the sequences were selected to contain anchors for two MHC class II antigens (Table I). Each of the peptides synthesized contained at least two of three anchors for each HLA-DR antigen, as shown in the Table I, and the main Pl anchors for most class II alleles. In peptides D122, F12, F7, F6, F8 and F13, positions P4 and P5 are occupied by hydrophobic, aromatic followed by aliphatic residues in that order to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides for MHC-class II molecules may differ from natural ligands because the latter incorporate constraints of processing in addition to binding requirements. For these reasons, peptides were synthesized by following, when possible, the common motifs for all MHC-class II molecules defined by pool sequencing of naturally processed peptides (7). This was possible for four peptides, F6, F7, F13 and F14. In these peptides, the sequence was extended to include Pro (P) N-terminal to either the Tyr (Y), which is the P1 anchor for HLA-DR1, DR3, DR4, and DQ7, or Trp (W) which is reported by the Pl anchor for HLA-DR4 and DR1 1. Peptides F6 and F7 overlap in the first thirteen residues. In F6 the sequence was extended at its C-terminal to incorporate a region 783-797 previously reported to induce proliferation of PBMC from breast cancer patients. The sequence of F14 was also extended to include Pro at the C terminus, after Arg (R), the third anchor in the correct position for HLA-DR3 and HLA-DR1 1. Sequences were also extended at the N- and C-termini. This was made to facilitate the natural proteolytic trimming of peptides since most aminopeptidases stop cutting one residue before reaching a Pro residue (7).

Stimulation and propagation of T cells. Freshly separated PBMC from healthy donors and ovarian cancer patients were stimulated with each HER-2 peptide at a final concentration of 50 µg/ml and cultured at 1×10^6 cells/ml in RPMI 1640 medium (GIBCO) with 5% pooled human AB serum and antibiotics (complete RPMI medium) (1). After 3 - 4 days of stimulation with each peptide, cultures were expanded with IA-2 (20 - 40 U/ml) for the following week (15, 16). To induce antigen specific T cells, the cells were then "rested" for 3 - 4 days by culture in

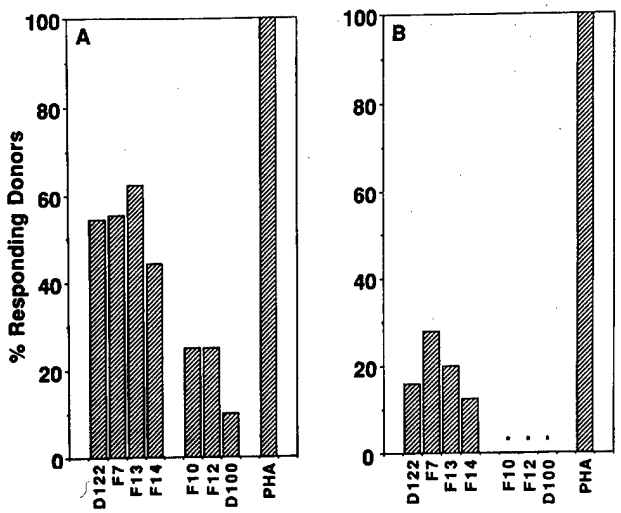


Figure 1. *Histograms of positive blastogenic responses of PBMC from (A) 14 healthy donors and (B) 25 ovarian cancer patients to seven HER-2 peptides of the sequences listed in Table I. The Y axis indicates the fraction of donors with a positive response to each peptide. A blastogenic response was considered significant when the p values for peptide stimulated vs. control cultures were <0.05. Stimulation indexes (S.I.) were obtained by dividing the means of cpm proliferation of PBMC in the presence of peptides with the means of cpm proliferation in the absence of peptide. S. I. for the peptides inducing significant proliferation ranged between 1.5- 3.0, while for the peptides that did not induce significant proliferation ranged between 0.9 - 1.2. Proliferation values (cpm + SD) are shown in Figures 2 and 3. Peptides F7 and F13 elicited the most positive responses in PBMC from both healthy donors and patients (at least 5 donors positive in each group). In (B), (*) the frequency of responses to F10, F12, and D100 respectively were as follows: F10: 1/25, F12, 1/25 and D100: 2/25.*

the absence of IL-2. Then, the cells were stimulated at a 1:1 (stimulator:responder ratio) with irradiated (10.000 Rad) autologous PBMC, which had been flrst stimulated with PHA, expanded in IL-2, and then were pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (16). Control cultures were stimulated with the same number of IL-2 expanded PBMC in the absence of peptides. For further expansion, four to five days later 20 - 40 U/ml IL-2 was added to the cultures for seven additional days.

Proliferation assays. The proliferation assay was done by culturing 2×10^5 PBMC from each donor in quadruplicate in a 96 well plate in 200 µl with each peptide at 50 µg/ml, with tetanus toxoid at 5 µg/ml and PHA (GIBCO) at a final concentration 1:100 for 96 h as described (1, 16). For the last 16 h, 1 µCi [^{3}H]-Tdr was added to the cultures. Afterwards, the cells were harvested and the radioactivity counted in a Beckman LS3501 liquid scintillation counter as previously described (6). A significant proliferative response was deflned as a statistically significant increase in the cpm proliferation in the tetraplicate cultures stimulated with any of the peptides, PHA, or TT, above that in cultures from the same donor that received peptide. Values obtained for cpm (^{3}H-Tdr incorporation) by the PBMC incubated with PBS, PHA or synthetic HER-2 peptides were examined by the Student's t test. Differences were considered significant when the P values were < 0.05.

Flow cytometry. T cells were tested in fluorescence experiments to determine the surface antigen expression as previously described (2, 6).

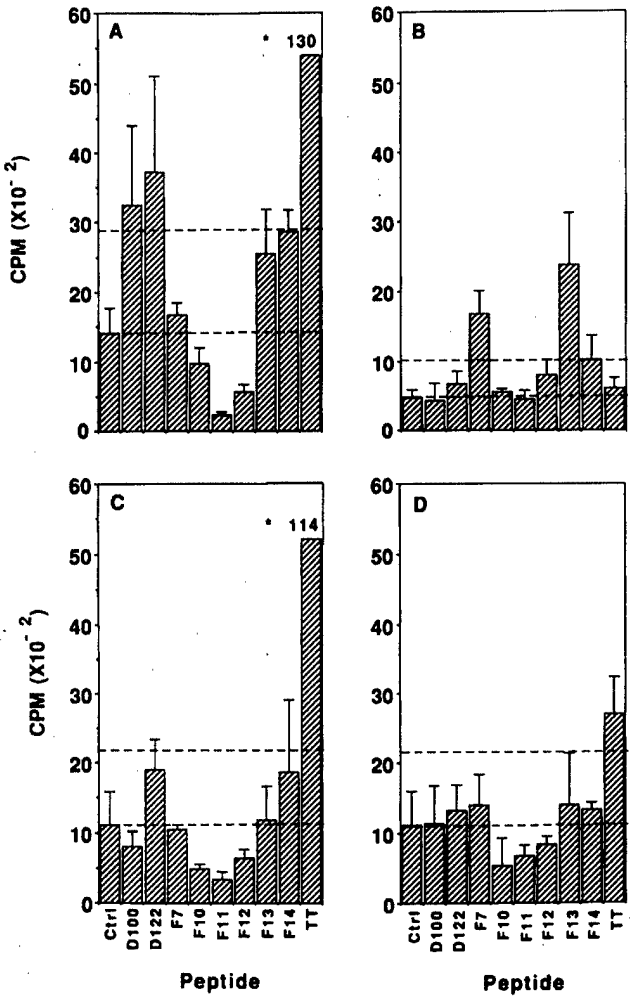


Figure 2. *Proliferative responses to HER-2 peptides by PBMC from four healthy donors A, B,C,D determined in the same experiment. Two responded to peptides as follows: Donor A to D 100, D 122, F13 and F14; Donor B to F7 and F13. Two were non-responders (panels C, D). Results indicate cpm ± SD. Dotted lines indicate proliferation corresponding to S.I. of 1.0 and 2.0 respectively. *cpm proliferation to TT not on scale.*

Surface antigen expression was determined by FACS analysis using a FACSscan (Beckton-Dickinson, Sunnyvale, CA)) with a log amplifier. CD3, CD4 and CD8 antigen expression on T cell cultures was determined by immunofluorescence with corresponding mAb FITC-conjugated (Beckton Dickinson).

Cytokine production. The ability of PBMC to produce antigen induced IFN-γ and IL- 10 was determined by culturing the PBMC either as unstimulated or stimulated with the corresponding peptides or PHA, (GIBCO) diluted 1:100, or tetanus toxoid. Supematants were collected after 48h and stored frozen at -20°C until assayed for cytokine level. Cytokine containing supematants from the T cell lines were generated by adding OKT3 and phorbol myristate acetate (PMA) to the cells to 96 well plate for 18h as described (17, 18). Afterwards, supernatants were collected for measurement of IFN-γ and IL-10 levels. IFN-γ and IL- 10 were measured by double sandwitch-ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). Supematants from tetraplicate wells were pooled and tested in triplicates. The cytokine assays were calibrated with human recombinant IFN-γ and IL-10 to detect each cytokine in the range of 10-1000 pg/ml.

Results

Proliferative responses of PBMC to HER-2 peptides. To map the HER-2 peptides most frequently recognized and to identify potentially immunodominant epitopes, we determined the responses to individual peptides of PBMC from twenty five ovarian cancer patients. Patients previously treated with chemotherapy were allowed on this study, since immunotherapy approaches to ovarian cancer are usually initiated after conventional therapies thus making the responses of these donors more likely to reflect the responder status of candidates for tumor-vaccine therapies. Responses of PBMC from healthy donors to the same peptides were tested in parallel in the same experiment. Depending on the number of PBMC available from each ovarian cancer patient a minimum of four peptides and PHA were tested in the same experiment. Results are summarized in Figure 1. Peptides Fl 1 (HER-2, 5-19) (which corresponds to the highly hydrophobic signal area) F6 (HER-2, 777-797) and F8 (HER-2, 832 - 851) were not easily solubilized in PBS, thus their use for stimulation was discontinued after several assays. PBMC from most donors were tested with at least six peptides in each experiment. Each donor responded to some peptides but not to others. The lack of a common response it is not entirely suggesting that these peptides do not bind MHC molecules. A negative response of any of these peptides could also reflect T cell unresponsiveness (tolerance) to this epitope.

To ensure that the lack of responsiveness of PBMC to HER-2 peptides did not reflect a generalized suppression of responses to antigen or mitogen, all patients' lymphocytes were tested for their ability to respond to PHA. Of 29 patients tested, 28 showed significant responses to PHA compared with unstimulated cultures. All PBMC samples from healthy donors showed significant responses to PHA (data not shown). Together these results indicated that most PBMC from the ovarian cancer patients with advanced disease respond to PHA after chemotherapy and that their ability or failure to respond to HER-2 peptides did not reflect their ability to respond to a T-cell mitogen.

The frequency of responses of healthy donor PBMC to four peptides: D 122, F7, F13 and F14 was higher than the frequency of responses to three other peptides, F10, F12, and D100. The same pattem of preferential responses to F7 and F13 was seen in PBMC from ovarian cancer patients (Figure lB). The results also indicate that PBMC from ovarian cancer patients responded at a significantly lower frequency than healthy donors to F7, F13, F14 and D122. However, the decrease was not the same for all peptides. For peptides D122, F13, and F14 the decrease in responses in PBMC from patients versus healthy donors was in the range of 70%, while for peptide F7 the decrease was only 50%. No significant

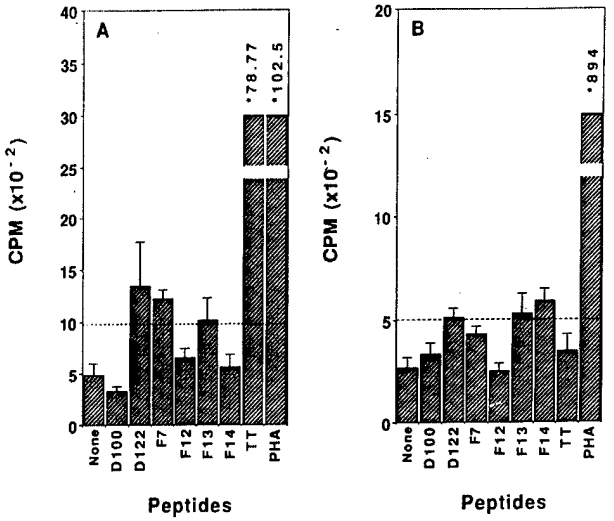


Figure 3. *Proliferative responses to HER-2 peptides of PBMC determined in the same experiment. (A) ovarian cancer patient; (B) healthy donor. Significant differences between peptide stimulated and no peptide cultures were observed in (A) for D122, F7 and F13 and in (B) for D122, F13 and F14. Dotted lines indicate proliferation corresponding to S.I. of 2.0.*

responses to peptides D100 (1086 - 1098), F10 (975 - 987), and F12 (449 465) were observed. Representative results from PBMC of four healthy donors (two responders and two non-responders) are shown in Figure 2. Donors A and B were considered responders based on the ability of their PBMC to proliferate to at least one of the eight HER-2 peptides tested. These responses were significantly higher than responses by PBMC that have not been pulsed with peptides. In both responders and nonresponders, the responses to HER-2 peptides did not correlate with the ability of the same PBMC to respond or with the magnitude of response to TT (Flgures 2 and 3). Of twenty flve ovarian cancer patients tested, seven responded to F7 with statistically signiflcant differences in cpm proliferation between peptide-induced and control cultures. Of seven F7 responding patients three responded to F13 two responded to F7, F13 and F14; one to F7 and F14; and four only to F7. Of five F13 responding patients two did not respond to F7 or Fl4. Only two patients responded to all four peptides: F7, F13, F14 and D122. There was no signiflcant difference in the magnitude of their proliferative responses to these four peptides.

For comparison, the pattem and the magnitude of the proliferative responses to HER-2 peptides of PBMC one of these patients (designated as Patient A) is shown in Flgure 3 together with those of PBMC from another healthy donor tested in the same experiment. Both donors responded to D122, F7, F13 and F14 but failed to respond to D100 and F12. To establish whether the pattern of responses to the HER-2 peptides in PBMC from ovarian cancer patients changed over time, responses to the same peptides were determined at two additional time points over five months when the patients were not receiving chemotherapy. PBMC from Patient A showed a constant high level of responses to at least two of the four peptides tested, but PBMC from a nonresponding patient failed to proliferate above the control levels. The same PBMC showed significant responses to PHA. It should be mentioned that sixteen months after the first determination, PBMC from the same Patient A responded primarily to F13 also less strongly to F7 (data not shown). PBMC from the same responding patients were tested again after several months when the disease progressed. At this time significant proliferative responses to HER-2 peptides were not observed. All three patients that responded to both F7 and F13 had stable disease. This suggests that disease progression affects the ability of PBMC from cancer patients to respond to HER-2 peptides.

In vitro expansion and cytokine production by HER-2 peptide stimulated T cells. To establish whether HER-2 peptide stimulated lymphocytes can be expanded in culture as T-cell lines, PBMC from Patient A were selected for these experiments. These PBMC were chosen because they showed a stable and significant proliferative response to at least four HER-2 peptides of distinct sequence. Primary cultures with F7, D122, F13 and F14 were initiated for four days, after which IL-2 (40 U/ml) was added for four more days. Afterwards, IL-2 was removed and the cells were "rested" in complete RPMI medium in the absence of IL-2 for four days. Afterwards, each peptide initiated culture was restimulated with autologous T cells from PHA-stimulated PBMC expanded in IL-2 prepulsed with the corresponding peptide. Control cultures were then restimulated with PHA blasts in the absence of peptides. After four days, IL-2 (20 U/ml) was added to the cultures for 48 h. The S.I. were determined by comparing the 3H-Tdr incorporation during the last 16 h. As shown in Figure 4A all peptide stimulated cultures showed an increase in S.I. of >2.0 over the control cultures. Similarly, cultures stimulated initially with TT, PHA and OKT3 in the presence of IL-2 showed a significant increase in proliferation over the peptidestimulated cultures.

To address whether T cells induced by one of the peptides can be stimulated to proliferate by another HER-2 peptide, we investigated the response of F13-induced T cells to F13, D122, F14, and F7. The results are shown in Figure 4B. PHA and OKT3 mAb were used for stimulation as positive controls. Cells were counted after four days. A signiflcant increase in number over control cultures was observed in F13 induced cultures restimulated with F13. In contrast, F13-induced cultures showed no signiflcant proliferation in response to F14, and F7 while in response to D122 the viable cell number actually decreased. These results indicate that F13-induced T cells preferentially proliferate in response to F13 and are only minimally cross-stimulated to proliferate by other HER-2 peptides.

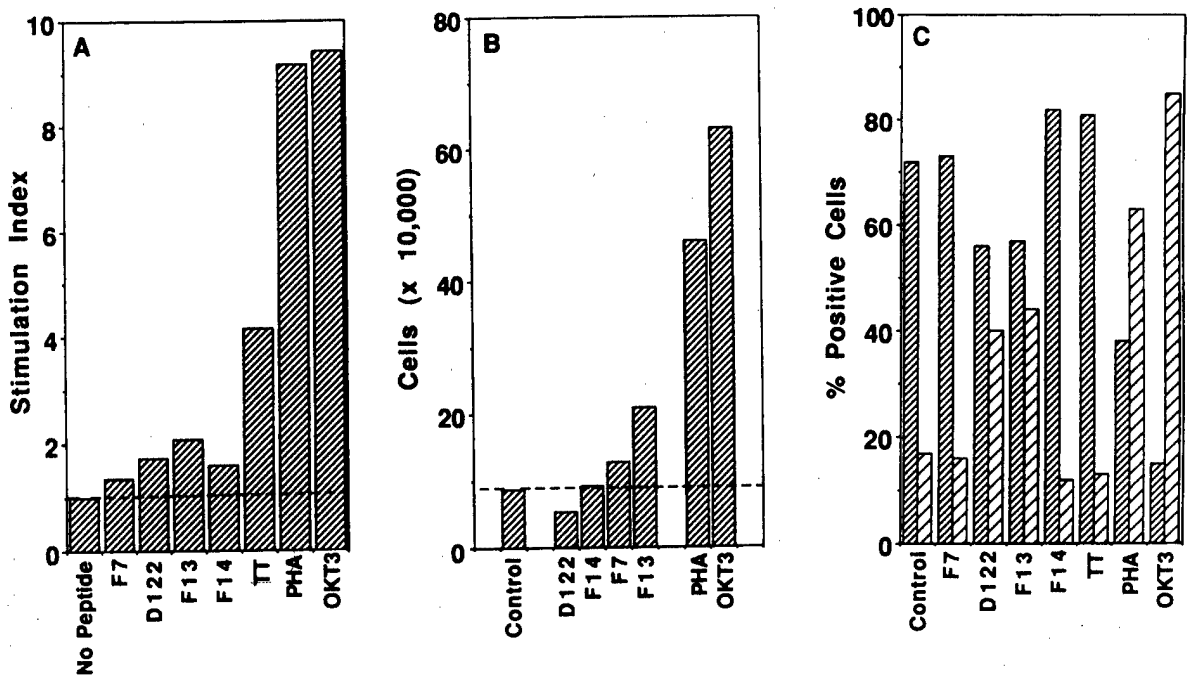


Figure 4. *(A) Proliferative responses to HER-2 peptides F7, F13, F14, and D122 by T-cell cultures induced with the corresponding peptide and expanded in IL-2. (B) Increase in cell number of F13 induced T cells described in (A) after restimulation with either no peptides (control), each of the peptides D122, F14, F7 and F13; or PHA and OKT3 mAb. Experimental conditions were as described in the Materials and Methods. (C) Cell surface phenotypes of peptide-induced cultures described in (A), after restimulation with each peptide and expansion in IL-2 ▨ $CD4^+$ cells, ▨ $CD8^+$ cells.*

To define the T cell phenotype, the resulting PBMC cultures, were analyzed for CD4 and CD8 antigen expression. Analysis of the phenotype of the control cultures was performed in parallel. The results are shown in the Figure 4C. Most cells in the primary stimulated cultures were $CD4^+$ T cells, however there were some significant differences between cultures. F7- and F14-stimulated cultures contained 12 and 16% $CD8^+$ cells respectively, while D122 and F13-stimulated cultures had a significantly higher proportion of $CD8^+$ cells (40 - 44%). Control cultures which were stimulated only with autologous PBMC and IL-2 were of predominantly of the CD4 phenotype while in PHA and OKT3 stimulated cultures $CD8^+$ cells were in majority. These results indicate that, for the same donor, stimulation with each peptide affected in a different way the proliferation of either $CD4^+$ or $CD8^+$ cells or both. This does not reflect the ability of one or another subset to proliferate better in the presence of IL-2. Control cultures which were not stimulated with exogenously added peptides, and TT-stimulated cultures showed a predominantly $CD4^+$ phenotype. Inhibition studies using anti-HLA-mAb indicated that HER-2 peptides induced proliferation was inhibited by anti-MHC class II and at lesser extent by anti-MHC-class I Abs suggesting that the responder cells are T cells (data not shown).

Each set of HER-2 stimulated lymphocytes showed different proportions of $CD4^+$ and $CD8^+$ cells. Since this may reflect the ability of each peptide to induce cytokines, which can affect the proliferation of each T cell subset, the capacity of each HER-2 induced T-cell line to secrete IFN-γ and IL- 10 was determined. These cytokines have been associated with Thl and Th2 types of responses, respectively (19-21). Since cells cultured in IL-2 usually produce background levels of IFN-γ, all peptide-stimulated T-cell lines and control lines were washed and cultured in complete RPMI medium without IL-2 for two days, before being stimulated with OKT3 and PMA. The results of one representative experiment (of two experiments performed) are presented in Figure 5.

T-cell lines stimulated by F7, F13, and F14 produced IFN-γ at higher levels than observed in control cultures stimulated with autologous PBMC but not with peptides. The highest levels were observed with F13 and were similar to the levels induced by TT-induced T cell lines. Interestingly, a different pattern of IL-10 secretion was observed. IL-10 secretion by F13-stimulated T cells was only slightly above the control levels. However, high levels of IL-10 were found in the supernatants from F7- and F14-stimulated T cells. The levels of IL-10 were almost half the level of IL-10 produced by TT-induced T cells. In contrast, while the levels of IFN-γ produced by D122-induced line were higher than those produced by the control cultures, the levels of IL-10 produced by the D122-induced line were minimal. These results show a good correlation between the IL-10 secretion and the $CD4^+/CD8^+$ ratio of T cells from HER-2 peptide induced T

cell lines. F7-, F14- and TTinduced T-cell lines secreted high levels of both IFN-γ and IL-10. Conversely, D122- and F13-stimulated cultures secreted different amounts of IFN-γ but low amounts of IL-10. This may be suggestive of a Thl function for the F13 peptide in this patient and for a Th2 function for the F7 peptide in the same patient.

Discussion

Recognition of HER-2 epitopes by $CD8^+$ cytotoxic T lymphocytes has been extensively documented (2-5). However significantly less information is available about the recognition of HER-2 epitopes by $CD4^+$ cells. Although $CD4^+$ cells may not be always involved in tumor lysis in breast and ovarian cancer, helper T cells may be essential for initiating, sustaining and amplifying an anti-tumor response. CTL induced by stimulation with Ag in the presence of co-stimulation with B7- may become exhausted by the interaction with B7- tumor cells. The presence of Ag specific $CD4^+$ T cells may provide the "self-help" iieeded to sustain CTL responses (22, 23). Thus Thl cells recognizing peptides derived from the processing of HER-2 may produce cytokines (IFN-γ, TNF-α/β) that are thought to provide help for CTL function. The same HER-2 enitones mav produce Th2 cytokines in association with other MHC-class II types.

The objectives of this study were to (a) determine whether HER-2 peptide recognition occurs in healthy donors and in ovarian cancer patients with advanced disease and (b) whether distinct HER-2 peptides differ in their ability to modulate the cytokine secretion potential of the T cells from the same donor. In this report we present evidence that T cells responsive to multiple epitopes on a self-protein, HER-2, exist *in vivo* in healthy donors as well as in ovarian cancer patients. These cells can be stimulated to proliferate and expand *in vitro* and can secrete Th1 and Th2 cytokines. The observed *in vitro* responses of normal T cells to multiple peptides derived from HER-2 cannot be attributed to mitogenic effects by a particular peptide since (a) different peptides elicited PBMC proliferation in different donors and (b) three peptides containing the same P1 anchor for the same MHC-class II molecules failed to induce proliferative responses with the same frequency as four other peptides. Our analysis revealed that PBMC from ovarian cancer patients after chemotherapy responded less frequently than PBMC from healthy donors to the same peptides.

The reasons for the reduced frequency of responses in PBMC from ovarian cancer patients are still unknown. One possibility, to be addressed in future studies, is whether the ability of T cells from patients to respond is affected by chemotherapy. Chemotherapy can eliminate proliferating reacting clones to HER-2 peptides. An alternative is that individuals susceptible to ovarian cancer may be less responsive to self-antigen. This hypothesis could be tested in future studies using HLA-typed ovarian cancer patients. At this moment there is unknown whether there is a

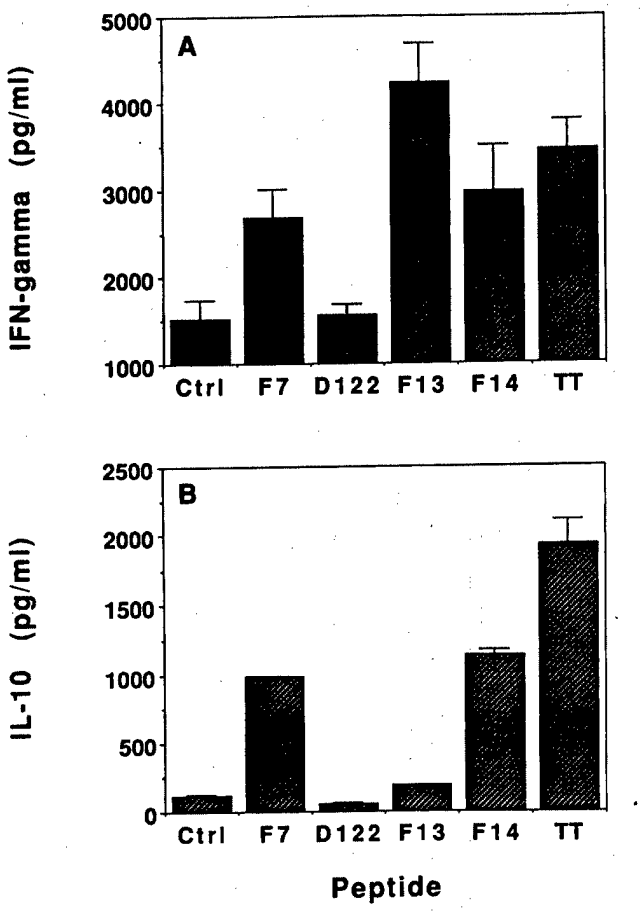


Figure 5. *Cytokine secretion by HER-2 peptides-induced T-cell lines. Equal numbers of cells (10^5) from each culture were plated in 96-well plates and incubated with PMA and OKT3 mAb as described in Materials and Methods. Cytokine expression was determined by ELISA. The concentrations were calculated by comparison with standard plots of IL-10 and IFN-γ in the same assays.*

disproportionate expression of MHC-class II allele in the cancer patients that may account for the different response pattern to the peptide antigens. Other possibilities currently under investigation are whether (a) $CD4^+$ T cells from these patients are anergic to these peptides or (b) their response is suppressed (1, 22). Suppressive effects due to disease progression may account for the lack of responses to these peptides at this stage. Although the group of patients with stable disease is relatively small to allow conclusive comparisions to be made, three of seven patients with stable disease responded to F13 while only two of eighteen patients with progressive disease (11%) responded to F13. In several cases when both PBMC and TAL were available from the same patient, we found that only PBMC responded to these peptides, suggesting the presence in situ of potentially negative regulatory mechanisms. Our preliminary results on the pattern of cytokine responses to F7 and F13 show that for a number of patients IL-10 was detectable at 48h in the

peptide induced cultures but the levels of IFN-γ were below the levels of detection of the assay (10 pg/ml). PHA stimulated PBMC from the same donors secreted both IFN-γ and IL-10. In other patients, only F7induced PBMC secreted TNF-α and/or IFN-γ (Melichar *et al* manuscript in preparation).

In our study, PBMC from ovarian cancer patients responded less frequently to F13 than did PBMC from healthy donors. The frequency of responses to F7 and F13 in healthy donors (54 and 62%, respectively) does not correlate with the frequency of expression of HLA-DR4 (25%) in the human population, suggesting that these peptides can be presented by other class II molecules.

Our current study also sought to elucidate the ability of HER-2-peptide stimulated T cells to expand and secrete cytokines. In this case we studies PBMC from a patient who had shown a stable response to several HER-2 peptides over a six month period. T cell lines of predominantly $CD4^+$ phenotype were readily expanded by restimulation with these peptides and low concentrations of IL-2. Interestingly, the resulting T cell lines differed in their proportions of $CD4^+$ and $CD8^+$ cells in their pattern of IFN-γ and IL- 10 secretion.

One possible explanation for these observed differences is that peptides F13 and F7 function as Thl and Th2 epitopes respectively in association with certain MHC-class II molecules. Both F7 and F13 contain a set of P1-P4-P6 anchors for HLA-DR4, though these sequences differ in charge at the P4 anchor: R (782) in F7 and E (892) in F13 correspond to the motifs for peptide binding sites to the DRBl*0402 and DRBl*0401/0404 alleles (12, 23). Phenotypic analysis of cells in the T cell lines stimulated by these peptides revealed a significantly larger population of $CD8^+$ cells in F13- than in F7- stimulated T cell lines from this donor. Furthermore, a T cell response to the epitope HER-2:783-797 mapped with the peptide SRLLGICLTSTVQ was detected in a breast cancer patient with high level of HER-2 auto-antibodies (1). F7, HER-2 (777-789) overlaps with HER-2:783797 in the area 777 - 783. The possibility that T cells stimulated by F7 can provide help for Ig synthesis deserves further consideration.

The fact that T cells from healthy donors and ovarian cancer patients respond to HER-2 peptides, indicates that tolerance to some of these self-epitope is not induced. Ongoing studies aim to determine whether T cells induced by these peptides recognize the HER-2 protein, the restriction element and the dominant epitopes for induction of a Th1 response. The implications of the observed responses in immunity to, or progression of ovarian cancer deserve further consideration as to whether the responses to these peptides correlate with HER-2 expression, stage and clinical outcome. Such studies are currently in progress in our laboratory. The results presented in this study should be useful for investigation of the mechanisms of Ag specific immunity, auto immunity, tolerance and design of epitope specific tumor vaccines.

References

1 Disis ML, Callenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB, Moe R, and Cheever MA: Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res *54:* 16-20, 1994.

2 Ioannides CG, Fisk B, Fan D, Biddison WA, Wharton JT, and O'Brian CA. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptides derived from the HER-2/neu proto-oncogene. Cell Immunol *151:* 225-234, 1993.

3 Yoshino I, Goedegeboure PS, Peoples GE, Parikh AS, DiMain JM, Lyerly HK, Gazdar AF, and Eberlein TJ: HER-2/neu derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer. Cancer Res *54:* 3387-3390. 1994.

4 Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, and Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc.Natl.Acad.Sci, USA *92:* 432-436, 1995.

5 Fisk B, Blevins TL, Wharton JT, and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu-proto-oncogene recognized by ovarian tumor specific CTL lines. J Exp Med *181:* 2709-2717, 1995.

6 Ioannides CG, Platsoucas CD, and Freedman RS: Immunological effects of tumor vaccines II. T cell responses directed against cellular antigens in the viral oncolysates. In Vivo *4:* 17-24.1990

7 Falk K, Rotzschke O, Stevanovic S, Jung G, and Rammensee H-G: Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constrainsts of processing, and general rules. Immunogenetics *39:* 230-242, 1994.

8 Kropshofer H, Max H, Muller CA, Hesse F, Stevanovic S, Jung G, and Kalbacher H: Self-peptide released from class II HLA-DR1 exhibits a hydrophobic two-residue contactmotif. J Exp Med *175:* 1799-1803, 1992.

9 Malcherek G, Falk K, Rotzschke O, Rammensee H-G, Stevanovic S, Gnau V, Jung G, and Melms A: Natural peptide ligand motifs of two HLA molecules associated with myastenia gravis. Int Immunol *5:* 1229- 1237, 1993.

10 Sette A, Sidney J, Oseroff C, del Guercio M-F, Southwood S, Arrheuius T, Powell MF, Colon SM, Graeta FCA, and Grey HG: HLA Drw4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. J Immunol *151:* 3136-3170, 1993.

11 Hammer J, Valsasnini P, Tolba K, Bolin D, Higelin J, Takacs B, and Sinigaglia F: Promiscuous and allele-specific anchors in HLA-DR binding peptides. Cell *74:* 197-203, 1993.

12 Hammer J, Gallazzi F, Bono E, Karr RW, Guenot J, Valsasnini P, Nagy ZA, and Sinigaglia F: Peptide binding specificity of HLA-DR4 molecules: Correlation with rheumatoid arthritis association. J Exp Med *181:* 1847-1855, 1995.

13 Sinigaglia F, and Hammer J: Motifs and supermotifs for MHC class II binding peptides. J Exp Med *181:* 449-451, 1995.

14 Marshall KW, Wilson KJ, Liang J, Woods A, Zaller D, and Rothbard JB: Prediction of peptide affinity to HLA-DRBl*0401. J Immunol *154:* 5927-5933, 1995.

15 Barker RN, and Elson CJ: Multiple self epitopes on the Rhesus polypeptides stimulate immunologically ignorant human T cells. Eur J Immunol *24:* 1578-1582, 1994.

16 Elson CJ, Barker RN, Thompson SJ, and Williams NA: Immunologically ignorant autoreactive T cells, epitope spreading and repertoire limitation. Immunol Today *16:* 71-76, 1995.

17 Barker E, Mackewicz CE and Levy JA: Effects of Thl and Th2 cytokines on $CD8^+$ cell response against human immunodeficiency virus: Implication for longterm survival. Proc Natl Acad Sci USA *92:* 11135-11139, 1995.

18 Tsuchida T, Parker KC, Turner RV, McFarland HF, Coligan JE, and Biddison WE: Autoreactive $CD8^+$ T-cell responses to human myelin. Proc. Natl Acad Sci USA *91:* 10859-10863, 1994.

19 Salgame P, Abrams JS, Clayberger C, Goldstein H, Convit J, Modlin RL, and Bloom BR: Differing lymophokine proflles of functional subsets of human CD4 and CD8 T cell clones. Science (Wash. DC) *254:* 279-282, 1991.

20 Matsuda M, Salazer F, Petersson M, Masucci G, Hansson J, Pisa P, Zhang, QJ, Masucci MG and Kiessling R: Interleukin 10 pretreatment protects target cells from tumor- and allele-specific cytotoxic T cells and downregulates HLA class I expression. J Exp Med *180:* 2371-2376, 1994.

21 Croft M, Certer L, Swain SL, and Dutton RW: Generation of polarized antigenspeciflc CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. J Exp Med *180:* 1715-1728, 1994.

22 Allison JP, Hurwitz AA, and Leach DR: Manipulation of costimulatory signals to enahnce antitumor T-cell responses. Curr Opin Immunol *7:* 682-686, 1995.

23 Wu T-C, Guarnieri FG, Staveley-O'Carroll KF, Viscidi RP, Levistsky HI, Hedrick L, Cho KR, August JT, and Pardoll DM. Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. Proc Natl Acad Sci, USA *92:* 11671-11675, 1995.

24 Topalian SL, Rivoltini L, Mancini M, Markus NR, Robbins PF, Kawakami Y, and Rosenberg SA: Human $CD4^+$ T cells speciflcally recognize a shared melanomaassociated antigen encoded by the tyrosinase gene. Proc Natl Acad Sci USA *91:* 9461-9465, 1994.

25 Nanda NK and Sercarz EE: Induction of anti-self-immunity to cure cancer. Cell *82:* 13-17, 1995.

26 Wucherpfennig KW, and Strominger JL: Selective binding of self peptides to diseaseassociated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases. J Exp Med *181:* 1597-1601, 1995.

27 Pfeiffler C, Stein J, Southwood S, Ketelaar H, Sette A, and Bottomly K: Altered peptide ligands can control CD4 T lymphocyte differentiation *in vivo.* J Exp Med *181:* 1569-1574, 1995.

28 Kumar V, Bheardwaj V, Soares L, Alexander J, Sette A, and Sercarz E: MHC binding affinity of an antigenic determinant is crucial for the differential secretion of IL4/5 or IFN γ by T cells. Proc Natl Acad Sci USA *92:* 9510-9514, 1995.

29 Wucherpfennig KW and Strominger JL: Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell *80:* 695-705, 1995.

30 Silver RF, Wallis RS, and Ellner JJ: Mapping of T cell epitopes of the 30-kDa α antigen of *mycobacterium bovis* strain bacillus Calmette-Guerin in purified protein derivative (PPD)-positive individuals. J Immunol *154:* 4665-4674, 1995.

31 Clerici M, and Shearer GM. The Th1 - Th2 hypothesis of HIV infection: new insights. Immunology Today *15:* 575-581, 1994.

33 Sheen-Chen S-M, Chou F-F, Eng H-L, and Chen W-J: An evaluation of the progr.ostic signiflcance of HLA-DR expression in axillary-node-negative breast cancer. Surgery *116:* 510-515, 1994.

34 Iarygin LM, Malyshev VS, Polianskaia IS, Denisov LE, Zimin II, Alekseeva PL, and Khotchenknoa NV. The clinical significance of determining the HLA-DR4 antigen in patients with breast cancer. Voprosy Onkologii *37:* 796-799, 1991.

35 Casoli C, Zanelli P, Adorni A, Starcich BR, and Neri, T: Serological and molecular study on the HLA phenotype of female breast cancer patients. Eur. J. Cancer *30:* 1207- 1208, 1994.

36 Lee JE, Reveille JD, Ross MI, and Platsoucas CD: HLA-DQBl*0301 association with increased cutaneous melanoma risk. Int. J. Cancer *59:* 510-513, 1994.

37 Apple RJ, Erlich HA, Klitz W, Manos MM, Becker TM, Wheeler CM: HLA DR-D9 association with cervical carcinoma show papillomavirus-type speciflcity. Nature Genetics *6:* 157- 162, 1994.

38 Takahashi T, Chapman PB, Yang SY, Hara I, Vijayasandhi S, and Houghton AN: Reactivity of autologous $CD4^+$ T lymphocytes against human melanoma. J Immunol *154:* 772-779, 1995.

39 Topalian SL, Rivoltini L, Mancini M: Human CD4+ T cells specifically recognize a shared melanomas-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. USA 91:9461-9465, 1994.

40 Becker JC, Brabletz T, Czemy C, Termeer C, and Brocker EB: Tumor escape mechanisms from immunosurveillance: induction of unresponsiveness in a specific MHC-restricted CD4+ human T cell clone by the autologous MHC class II+ melanoma. International Immunology 5: 1501- 1508, 1993.

41 Matsuo H, Batocchi A-P, Hawke S, Nicolle M, Jacobson L, Vincent A, Newsom-Davis J, and Willcox N: Peptide-selected T cell lines from mysastenia gravis patients and controls recognize epitopes that are not processed from whole acetylcholine receptor. J Immunol *155:* 3683-3692, 1995.

42 Ioannides CG, and Freedman RS: T cell responses to ovarian tumor vaccines. International Reviewsof Immunology *7:* 349-364, 1991.

43 Dong R-P, Kamikawaji N, Toida N, Fujita Y, Kimura A and Sasazuki T. Characterization of T cell epitopes restricted by HLA-DP9 in streptococcal M 12 protein. J Immunol *154:* 4536-4545, 1995.

Received July 25, 1996
Accepted September 10, 1996

Proliferative and Cytokine Responses to Class II HER-2/neu-associated Peptides in Breast Cancer Patients[1]

Todd M. Tuttle, Brett W. Anderson, William E. Thompson, Jeffrey E. Lee, Aysegul Sahin, Terry L. Smith, Kenneth H. Grabstein, J. Taylor Wharton, Constantin G. Ioannides,[2] and James L. Murray[2]

Departments of Surgical Oncology [T. M. T., W. E. T., J. E. L.], Gynecologic Oncology [B. W. A., J. T. W., C. G. I.], Breast Medical Oncology and Bioimmunotherapy [J. L. M.], Pathology [A. S.], and Biomathematics [T. L. S.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Corixa Corporation, Seattle, Washington 98104 [K. H. G.]

ABSTRACT

Previous studies have characterized the reactivity of CD8+ CTLs with ovarian and breast cancer. There is little information about the antigens and epitopes recognized by CD4+ T cells in these patients. In this study, we analyzed the ability of T cells from peripheral blood mononuclear cells of breast cancer patients to recognize HER-2/neu (HER-2) peptides. We found that 13 of 18 patients responded by proliferation to at least one of the HER-2 peptides tested. Of these peptides, one designated G89 (HER-2: 777–789) was recognized by T cells from 10 patients. Seven of nine responding patients were HLA-DR4+, suggesting that this peptide is recognized preferentially in association with HLA-DR4. Analysis of the specificity and restriction of the cytokine responses to G89 by G89-stimulated T cells revealed that these cells secreted significantly higher levels of IFN-γ than interleukin 4 and interleukin 10, suggesting priming for a Th0-T helper 1 response. The same pattern of cytokine responses was observed to the intracellular domain of HER-2 protein, suggesting that G89-stimulated T cells recognized epitopes of the HER-2 protein in association with HLA-DR4. Because HLA-DR4 is present in 25% of humans, characterization of MHC class II-restricted epitopes inducing Th0-T helper 1 responses may provide a basis for the development of multivalent HER-2-based vaccines against breast and ovarian cancer.

INTRODUCTION

Studies in animal models have demonstrated a significant role for T lymphocytes in antitumor immunity and have shown that CD8+ and CD4+ cells can mediate tumor rejection (1, 2). In recent years, significant emphasis has been placed on identifying epitopes recognized by tumor-reactive CD8+ CTLs. A remarkable feature of these Ags[3] is that they are nonmutated self proteins (3). This raises the possibility that CD4+ cells recognizing epitopes on the same self proteins in the context of MHC I and MHC II may also be present in cancer patients (4). CD4+ cells may either express direct killing or play a regulatory role in the differentiation of other CD4+ cells and of tumor-reactive CTLs (5).

The known repertoire of tumor Ag recognized by CD4+ cells is limited. There is little information on the restriction elements operating in each Ag system and the nature of responses (Th1/Th2) induced by self peptides activating CD4+ cells. Topalian *et al.* (6, 7) have identified HLA-DR4.1 (HLA-DRB1*0401)-restricted tyrosinase peptides that stimulated Th1 cytokine secretion by CD4+ melanoma TILs. This ability was shown to be dependent on the binding affinity of the peptide to HLA-DR (7). Yoshino *et al.* (8) have shown that CD4+ TILs secrete Th1 cytokines when presented with heat shock proteins associated with HLA-DR, suggesting that they may recognize peptides complexed to such proteins (8). CD4+ CTLs were shown to recognize a shared HLA-DR15 melanoma-associated Ag (9). MHC class II-restricted Th1 cytokine secretion by long-term cultured CD4+ TILs has also been reported in breast cancer patients, suggesting a HLA-DR4-associated response (10). Finally, autologous tumor-specific CD4+ CTLs have also been demonstrated in sarcoma restricted by HLA-DR4 and HLA-DR15 (11).

These studies have shown that *in vitro* cultured CD4+ cells of TILs can recognize class II-associated Ag. There is little information on the ability of class II-associated peptides from these Ags to induce and restimulate a response in healthy donors and patients with cancer. Proliferative responses of PBMCs reflecting responses by T cells to mutated Ras protein and peptides carrying the same mutation have also been detected in pancreatic and colon cancer patients vaccinated with the corresponding peptide (12, 13). In contrast, responses to w.t. or

Received 12/17/97; revised 4/28/98; accepted 5/15/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by DAMD Grant 17-97-I-7098 (to C. G. I.) and the Nellie Connally Breast Cancer Research Fund (to J. L. M.). Peptide synthesis was supported in part by Core Grant CA16672.

[2] To whom requests for reprints should be addressed, at Department of Gynecologic Oncology, Box 67 (C. G. I.), or Department of Bioimmunotherapy, Box 02 (J. L. M.), The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-4561.

[3] The abbreviations used are: Ag, antigen; PBMC, peripheral blood mononuclear cell; HER-2, HER-2/neu proto-oncogene; S.I., stimulation index; APC, antigen-presenting cells; HA, influenza hemagglutinin; w.t., wild type; ICD, HER-2 intracellular domain; IL, interleukin; TIL, tumor-infiltrating lymphocyte; Th1, Th0-T helper 1; P1, position 1; PHA, phytohemagglutinin; NO, no peptide.

mutated Ras have not been found in healthy donors (12). However, the immunity directed against mutated tumor proteins may be targeted to w.t. epitopes (14, 15). Breast cancer patients developed anti-p53 antibodies and T cells that proliferated *in vitro* in response to w.t. p53 only when mutated p53 accumulated in their tumors (15), suggesting that the enhanced presentation of w.t. p53 was leading to a Th2 response. Similarly, a breast cancer patient with HER-2 overexpression (HER-2^{hi}) and anti-HER-2 antibodies developed T cells that proliferated in response to both HER-2 protein and short HER-2 peptides (16).

These results suggest *in vivo* priming by enhanced presentation of self peptides due to HER-2 protein overexpression. There is little information on the ability of CD4+ cells from healthy donors and from cancer patients who do not overexpress HER-2 (HER-2^{lo}) to respond to HER-2 peptides. This is important because identification of CD4+ cells reacting with self peptides may allow not only identification of "protective" tumor Ag, but allow optimization of design of tumor vaccines, by incorporating a "self helper" peptide(s) that can amplify and spread a Th1 response when the disease progresses.

We recently found that healthy donors responded with higher frequency than ovarian cancer patients to a number of HER-2 peptides. We hypothesized that CD4+ T cells recognizing HER-2 are not deleted from the immune repertoire of healthy individuals (17). Because the patients in that study were not HLA typed and had advanced disease, we decided to investigate the ability of HER-2 peptides to induce proliferative responses in healthy patients with primary breast cancer of defined MHC class II type. We focused on two HER-2 peptides that induced the most frequent responses in our previous studies. We found that peptide G89 (HER-2, 777–789) induced responses with higher frequency (10 of 18, 56%) in this group and significantly higher in the HLA-DR4+ patients (7 of 9, 78%) than the other peptides tested. There was no difference in the pattern of cytokine responses between one patient who overexpressed HER-2 (HER-2^{hi}) and one healthy donor who did not overexpress HER-2 (HER-2^{hi}), suggesting that the ability of patients with localized breast cancer to respond to G89 it is not affected by HER-2 overexpression.

MATERIALS AND METHODS

Subjects. PBMCs were obtained from 18 breast cancer patients and 6 healthy volunteers (three DR4+ and three DR4−). All patients, with one exception, were clinically free of tumor at the time of study. Of the healthy volunteers, three were HLA-DR4+, and the others were HLA-DR4− (*i.e.*, MHC II phenotype was: donor 4, DR7, 11, DQ 2, 6; donor 5, DR13, 14; donor 6, DR11, 15, DQ6, 7). Eleven patients had pathology stage I disease, 5 had stage II, and 1 had stage III. One patient (patient 16) had no primary tumor yet was classified as having breast cancer because of the presence of a positive lymph node. All except one patient had undergone surgery and were free of disease at the time of study. One patient had recurrent disease. All patients except one had 0–3 positive lymph nodes. Tumor from only one patient had Black's nuclear grade III (advanced pathological characteristics); the remainder were grade I or II.

HLA Class II Molecular Oligotyping. Genomic DNA extracted from PBMCs as described (18–22) served as the substrate for amplification of a polymorphic locus-specific fragment of the HLA class II gene by PCR. For the *-DQB1* and *-DRB* loci, the flanking primers used were as follows: DRB-AMP-A, 5′CCCCACAGCACGTTTCTTG; DRB-AMP-B, 5′CCGCTGCACTGTGAAGCTCT; DQB-AMP-A, 5′CATGTGCTACTTCACCAACGG; and DQB-AMP-B, 5′CTGGTAGTTGTGTCTGCACAC.

Because of the large number of *HLA-DRB* alleles and the numerous shared sequences between different alleles, *HLA-DRB* typing was carried out in a stepwise manner. First, group-specific *HLA-DR* typing was performed using the primers DR-AMP-A and DR-AMP-B. Oligonucleotide typing of this PCR-amplified DNA allowed discrimination of *HLA-DR1, -DR2, -DR3/6, -DR4, -DR5 (-DRw11), -DR7, -DR8/12, -DR9, -DR10, -DR52a, -DR52b/c*, and *-Drw53*. Because there are numerous variants of *HLA-DR1, -DR2, -DR4, -DR5 (-DRw11), -DR6, -DR8/12*, and *-DR52b/c*, further discrimination of these subtypes required a second PCR using group-specific primers plus DRB-AMP-B. They include DRB-AMP-1 for the *HLA-DR1* group, DRB1-AMP-2 or DRB5-AMP-2 for the *HLA-DR2* group, DRB-AMP-3 for the *HLA-DR3, -DR5, -DR6, -DR8, -DR12* group, DRB-AMP-4 for the *HLA-DR4* group, and DRB-AMP-52 for the *HLA-DRB3* genes of the *HLA-DRw52* group. The sequences of the primers were as follows: DRB-AMP-1, 5′TTCTTGTGGCAGCTTAAGTT; DRB1-AMP-2, 5′TTCCTGTGGCAGCCTAAGAGG; DRB5-AMP-2, 5′CACGTTTCTTGCAGCAGGA; and DRB-AMP-4, 5′GTTTCTTGGAGCAGGTTAAC.

For *HLA-DRw52*-associated *-DRB1* genes (*HLA-DR3, -DR5, -DR6, -DR8*, and *-DR12*), the sequences of the primers were as follows: DRB-AMP-3 (5′CACGTTTCTTGGAGTACTCTAC), *HLA-DRw52*, and DRB-AMP-52 (5′CCCAGCACGTTTCTTGGAGCT).

PCR products separated by electrophoresis were blotted to Hybond N+ membranes (Amersham Pharmacia Biotech, Arlington Heights, IL) hybridized with [γ^{32}P]ATP-labeled allele-sequence-specific oligonucleotide probes. *HLA-DQB1* alleles were determined by hybridization with probes corresponding to variable sequences around positions 23, 26, 37, 45, 49, 57, and 70 of the *HLA-DQB1* outermost domain. "Broad" *HLA-DR* groups [*HLA-DR1, -DR2, -DR3/6, -DR4, -DR5 (11), -DR12, -DR7, -DR8, -DR9, -DR10, -DRB3*0101, -DRB3*0201-*0301, -DRB4*0101 (-DR53)*] were determined by hybridization with oligonucleotide probes corresponding to variable sequences around positions 10, 28, and 37 of the *HLA-DRB1* outermost domain. Subtypes of *HLA-DR1, -DR2, -DR3/5/6/8/12, -DR4*, and *-DRw52* were determined by hybridization of the respective group-amplified DNA to oligonucleotides corresponding to variable sequences around positions 28, 37, 57, 70, and 86 of the *HLA-DRB1* outermost domain.

HER-2 Peptide Selection. Peptides tested were selected if they contained the T-cell sites in HER-2 predicted by the computer program ANT.FIND.M, the general binding motif for human class MHC II Ag, and the anchors for a number of MHC class II Ags (HLA-DR1, -DR3, -DR4, -DR11, and -DQ7; Refs. 23–28), the sum of whose allelic frequencies covers 75–100% of Americans. The general peptide binding motif for various human MHC class II molecules consists of a P1 anchor, *i.e.*, an aromatic or large aliphatic residue in the first 3–5 amino acids

Table 1 HER-2 peptides used in this study

The Tyr and Trp italicized in position 3 or 4 may constitute P1 anchors. Similarly, the Val, Leu, and Met italicized in positions 4 and 5 may also constitute P1 anchors.

Peptide code	Position	Sequence															
HA	307–319		P	K	*Y*	*V*	K	Q	N	T	L	K	L	A	T		
F12	449–464	G	I	S	*W*	*L*	G	L	R	S	R	E	L	G	S	G	L
G88	450–462		I	S	*W*	*L*	G	L	R	S	R	E	L	G	S		
F14	474–487	T	V	P	*W*	D	Q	L	F	R	N	P	H	Q	A		
F7	776–788	G	S	P	*Y*	*V*	S	R	L	L	G	I	C	L			
G89	777–789		S	P	*Y*	*V*	S	R	L	L	G	I	C	L	T		
F13	884–899	V	P	I	K	*W*	*M*	A	L	E	S	I	L	R	R	R	F
G90	886–898			I	K	*W*	*M*	A	L	E	S	I	L	R	R	R	

close to the NH_2 terminus, and other major but less essential anchors at P4, P5, P7, and P9, counting from the P1 anchor (26–28). Because many peptides are capable of binding to many different MHC class II molecules because their sequences contain overlapping binding motifs for MHC class II molecules (27, 28), each of the peptides synthesized contained at least two of three anchors for each HLA-DR Ag and the main P1 anchors for most class II alleles (Table 1). In peptides G88, G89, and G90, positions P3 and P4 are occupied by hydrophobic, aromatic, and aliphatic residues, in that order, to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides may differ from those of natural ligands because the latter incorporate processing constraints in addition to binding requirements. Thus, peptides were synthesized by following, when possible, the common motifs for all MHC class II molecules defined by pool sequencing of naturally processed peptides (25). In F7, F13, and F14, the sequence was extended to include Pro N-terminal to either the Tyr (the P1 anchor for HLA-DR1, DR3, DR4, and DQ7) or the Trp (reportedly as the P1 anchor for HLA-DR4 and DR11). For comparative studies of the responses associated with HLA-DR4, 13-mer analogues of F12, F7, and F13 (*i.e.*, G88, G89, and G90, respectively) were selected, using the anchor alignment matching the standard HLA-DR4/DR1 helper epitope, HA peptide (HA: 307–319) (Table 1). The predicted binding affinities of these peptides for HLA-DR4 (as IC_{50}) according to Rothbard's algorithm were as follows: HA, 35 nM; G88, 180 nM; G89, 987 nM; and G90, 219 nM (29). Peptides were prepared by the Synthetic Antigen Laboratory of the M. D. Anderson Cancer Center, using a solid-phase method as described previously (17, 30). Their identity was determined by amino acid analysis. Their purity was 93–97% as determined by HPLC. Peptides were dissolved in PBS, aliquoted at 2 mg/ml, and stored frozen at −20°C until use. The codes used to identify HER-2 peptides were assigned by the Synthetic Antigen Laboratory.

Recombinant ICD. The ICD (Lys^{676}–Val^{1255}) was cloned by PCR from c-erbB-2 cDNA (provided by Dr. Jacalyn Pierce, National Cancer Institute). The ICD was expressed in *Escherichia coli* using a pET vector with an amino-terminal His tag. Recombinant ICD was purified from inclusion bodies by a combination of Ni^{2+} affinity chromatography, size exclusion, and ion exchange chromatography. The purified ICD was greater than 95% pure as judged by PAGE and Western analyses (data not shown).

Stimulation and Propagation of T Cells. Freshly harvested PBMCs from breast cancer patients and healthy volunteers were isolated by Ficoll/Hypaque centrifugation. CD4+ and CD8+ cells were isolated from the plastic nonadherent fraction with magnetic beads. Cells were cultured at 1×10^6 cells/ml in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% pooled human AB serum and antibiotics in 2 ml in each well of a 24-well plate (complete RPMI medium). HER-2 peptides were added at a final concentration of 25 μg/ml. In other wells, PBMCs were stimulated with 25 μg/ml HA peptide, PHA at a final concentration of 1:100, or medium alone. After 6 days of stimulation with each peptide, cultures were expanded with IL-2 (Cetus) at 20 units/ml for the following week (17, 31). To induce Ag-specific T cells, the cells were then "rested" for 3–4 days by culture in the absence of IL-2. Then, the cells were stimulated at a 1:1 stimulator:responder ratio with irradiated (10,000 Rad) PBMCs and pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (17). For expansion, 4–5 days later, 20 units/ml IL-2 was added to the cultures for 7 additional days. Surface Ag expression was determined by fluorescence-activated cell sorting analysis using a FACScan (Becton Dickinson, Sunnyvale, CA) with a log amplifier as described (17).

Proliferation Assays. For proliferation assays, a 100-μl aliquot was removed from each well of the 24-well plate of primary cultures after 4–6 days, as described (16). Quadruplicate samples were cultured in a 96-well plate with 1 μCi of [^{3}H]Tdr in a final volume of 200 μl. The cells were harvested 16 h later, and the radioactivity was counted in a Beckman LS3501 liquid scintillation counter (16). A proliferative response was defined as positive when differences in cpm values between cultures that received peptides and cultures that did not receive peptides were significant by the unpaired Student's *t* test ($P < 0.05$).

Cytokine Production. The ability to secrete IFN-γ, IL-4, and IL-10 was determined by culturing the PBMCs with the corresponding peptides. Supernatants were collected at different times and stored frozen at −20°C. The cytokine concentrations were measured by double sandwich ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). The cytokine assays were calibrated with human recombinant IFN-γ, IL-4, and IL-10 to detect each cytokine in the range of 15–1000 pg/ml. The following homozygous B cell lines were obtained from the American Society for Histocom-

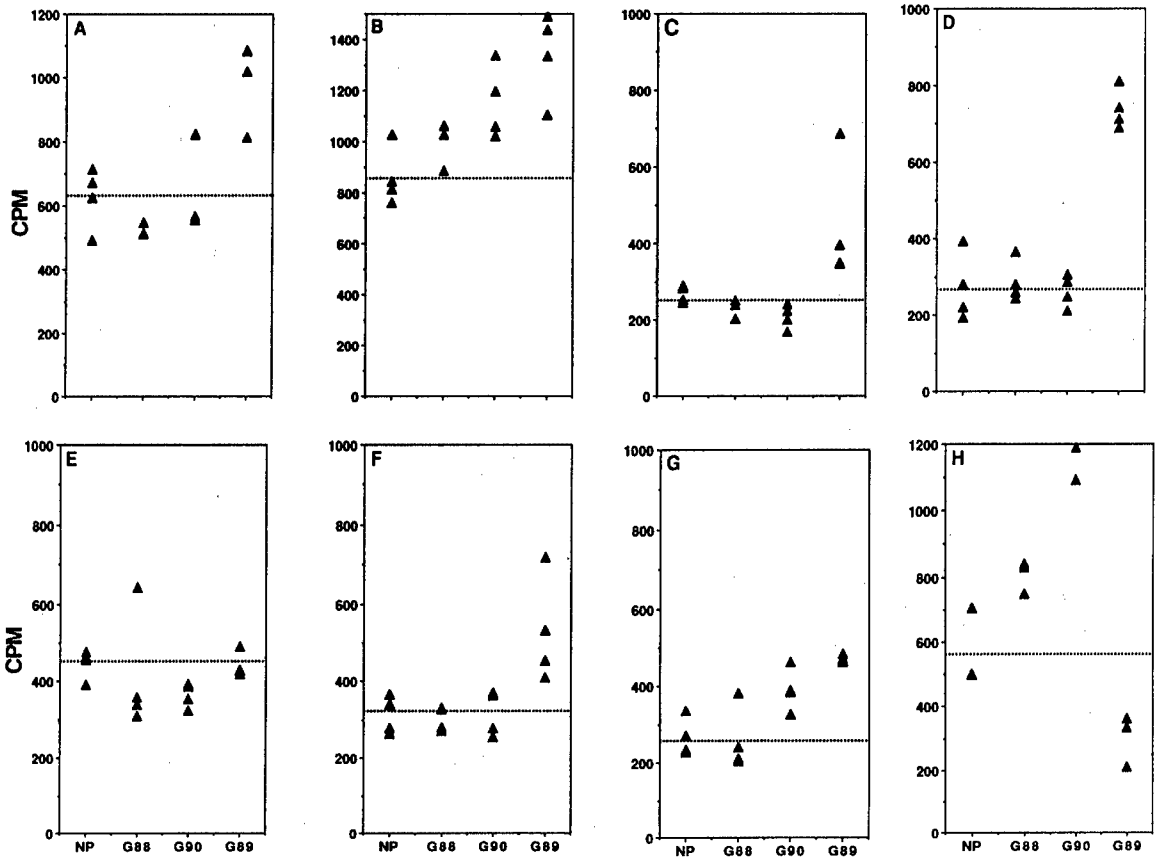


Fig. 1 Histograms of selected representative patterns of proliferation for eight breast cancer patients. *A*, patient 13, average NP value, 625 cpm; *B*, patient 7, average NP value, 862 cpm; *C*, patient 2, average NP value, 268 cpm; *D*, patient 9, average NP value, 272 cpm; *E*, patient 15, average NP value, 447 cpm; *F*, patient 12, average NP value, 313 cpm; *G*, patient 1, average NP value, 268 cpm; *H*, patient 5, average NP value, 568 cpm. Each determination was performed in quadruplicate; cpm for each of the replicates are represented by one triangle. Patients in *A–F* are HLA-DR4+ and those in *G* and *H* are HLA-DR4−. Responses to G89 were considered positive in *A–D* and *F* (HLA-DR4+ patients) and in *G* (HLA-DR4− patient) because in each of these donors tested, all cpm quadruplicate values in response to G89 were higher than each of the quadruplicate values of the control cultures unstimulated with peptide. Freshly isolated PBMCs from each donor were stimulated with peptides at a final concentration of 25 μg/ml. Responses were determined in 100-μl aliquots of cells removed from cultures on days 4–6 and tested for proliferation. Responses are shown for cultures stimulated for either 4 or 5 days. In most instances, significant proliferation was observed on two consecutive days (days 4 and 5 or days 5 and 6). An exception was made for patients 9 (*D*) and 15 (*E*), who showed responses only on days 5 and 6, respectively; patient 9 was considered a responder because the S.I. was >2.5, but patient 15 was considered a nonresponder because the S.I. to G89 was <2.0.

patibility and Immunogenetics Repository (Baltimore, MD) and used as APC for cytokine secretion: E418 (DRB1*1502, DRB5*0102, DQA1*0102, DQA1*0103, and DRB1*0601) and WT51 (DRB1*0401, DRB4*0101, DQA1*0301, and DQB1*0302).

Statistical Methods. Differences in proliferative responses were analyzed using Student's *t* test for unpaired samples. Differences in frequency for class II alleles were assessed using the Cochran Q test (32).

RESULTS

Recognition of HER2 Peptides in Breast Cancer Patients. PBMCs of breast cancer patients were cultured with HER-2 peptides or medium alone for 4–6 days. To ensure that lack of responsiveness of PBMCs to any of HER-2 peptides did not reflect a generalized suppression of responses to Ag or mitogen, all subjects' lymphocytes were tested for their ability to respond to PHA. Because responses to F7 (HER-2, 776–788) and F13 (HER-2, 884–899) were previously observed with higher frequency in healthy individuals (17), we wanted to address the question of the ability of the T cells from breast cancer patients to respond to HER-2 peptides, in association with certain MHC class II types, expression of HER-2 in their primary tumor, and the lymph node status. Because the patients were tested in the order they presented and not based on their HER-2 expression, when the S.I. was <2.0, to increase the sensitivity of detection, responses were considered positive when the cpm in each of the quadruplicate cultures stimulated with peptides was higher than each of the quadruplicate control cultures on two consecutive days (days 4 and 5 or days 5 and 6). This approach allowed us to identify responders without arbitrary cutoffs, using S.I.

Emphasis was given to HLA-DR4+ patients because of the recently reported association of HLA-DR and HLA-DR4 with

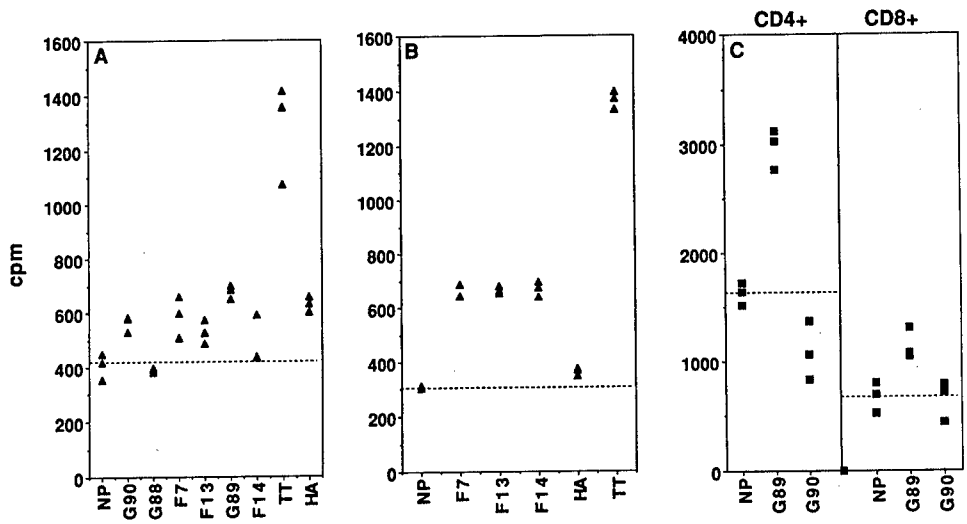


Fig. 2 Proliferative responses to HER-2 peptides by PBMCs from patient 13 (*A*; designated R; ▲), PBMCs from healthy donor 2 (*B*; ▲) determined in the same experiment with patient 13, and CD4+ and CD8+ cells from healthy donor 3 (*C*; designated L; ■). Both donors 2 and 3 were HLA-DR4+. The MHC class II phenotypes of the three healthy donors tested were as follows: donor 1, DR4, 13 (w52), DQ1, 3; donor 2, DR4, 15 w53, DQ6, 7; and donor 3, DR4, 13 w52, DQ1, 3. *A* and *B*, responses were determined 5 days after incubation with peptides in the same experiment for both donors. Responses in donor 2 were determined only to peptides F7, F13, and F14. Results similar to those of donor 2 were obtained for donor 1. *A* and *B*, *TT*, tetanous toxoid. See Table 2 for responses to HA peptide. *C*, responses were determined after 4 days of incubation with peptide, including the last 16 h the presence of ^{3}H-Tdr. Equal numbers of autologous plastic-adherent PBMCs were used as APC.

favorable prognosis in breast cancer (33, 34). Because both F7 and F13 contain HLA-DR4 anchors but differ in the length and position of the anchor motifs, we synthesized two 13-mers, designated G89 and G90. Each contained a hydrophobic aromatic followed by a hydrophobic aliphatic residue at P3 and P4 (Table 1). A control peptide of the same length: G88 (HER-2, 450–462) based on the sequence of F12 (HER-2, 449–464) was prepared (Table 1) because it had the same pattern of residues in P3 and P4 as G89 and G90. G88 was chosen as a control because responses to F12 were previously observed only infrequently (17). The predicted HLA-DR4 binding affinity of G88 was similar to that of G90 but significantly lower than that of G89.

The responses to HER-2 peptides G89 and G90, together with the responses to control peptide G88 for 8 of the 18 patients tested (including responders and nonresponders), are shown in Fig. 1. In all six G89 responding patients shown (Fig. 1, *A–D*, *F*, and *G*), the cpm values in all replicate cultures were higher than the cpm values in each replicate in PBMC cultures from the same patient that had not been stimulated with exogenously added peptide (NP). In these six patients, the cpm values in the replicate cultures stimulated with the control G88 peptide of higher binding affinity to HLA-DR4 than G89 were not significantly different from the cpm values in the NP cultures. This was confirmed by the fact than in the two nonresponders (Fig. 1, *E* and *H*), the cpm values in the replicate cultures stimulated with G89 were not higher than the cpm values in cultures stimulated with G88.

To verify the stimulatory ability of G89 in comparison with control peptide G88 and NP, the experiments were repeated with another patient (No. 13). Both peptides G89 and G90 and their counterparts F7 and F13 were tested in the same experiment. The results (Fig. 2*A*) show that responses to control peptide G88 were not significantly different from the NP cultures. Responses to G89 and G90 were significantly different from responses to NP or G89, but they were not significantly higher than responses to F7 and F13. The ability of PBMCs of HLA-DR4+ healthy volunteers to recognize HER-2 peptides was also tested. Significant proliferative responses by the same criteria were detected in HLA-DR4+ individuals after primary stimulation of PBMCs with various HER-2 peptides, of which responses to two donors (No. 2 and 3) are shown (Fig. 2, *B* and *C*). CD4+ cells responded to G89 (Fig. 2*C*). Thus, the ability to recognize sequences of the HER-2 protein is within the realm of the T-cell receptor of healthy volunteers, as we reported (17).

Proliferative responses to HER-2 peptides from all 18 patients tested are summarized in Table 2. G89 and F7 were recognized by PBMCs from 10 and 8 patients, respectively. PBMCs from six patients recognized both G89 and F7. Responses to G90 and F13 were observed in six and three patients, respectively. The results show a higher frequency of responses for G89 and G90 containing MHC class II anchors in P3 and P4 than for their analogues (F7 and F13) with the anchors shifted. The frequency of responses to G89 was significantly higher than to other peptides ($P = 0.02$). The results also show preferential association (7 of 9) of the responses to G89 with the presence of HLA-DR4 ($P = 0.01$). Of the other alleles that were represented, four of five HLA-DR3 patients responded to G89, but three of four responders were also HLA-DR4+. Four of six HLA-DR2+ patients responded to G89, but three of four responders were also HLA-DR4+. The other three HLA-DR4− donors responded preferentially to F13, with S.I. values of 1.9, 1.7, and 1.5, respectively, but no significant proliferation to G89 was observed (data not shown).

HER-2 staining for the autologous breast tumors was performed by immunocytochemistry. HER-2 was overexpressed

Table 2 Summary of proliferative responses of breast cancer patients to HER-2 peptides

Significant proliferative responses according to Student's *t* test are designated +. Responses not significantly different from those in control are designated −. All patients tested showed significant proliferation to PHA (data not shown). The allelism of the HLA-DQ has been determined and is listed. Values for control cultures that were not stimulated with peptides (NP) are listed as C.

Patient	DRB	DRB	DQB	DQB	NP	HA	G90	G88	PHA	F7	F13	G89	F14
1	3	11	301	201	C	−	+	−	+	+	+	+	+
2	4	7	301	201	C	−	−	−	+	+	−	+	−
3	2	4	303	602	C	−	−	−	+	−	−	−	−
4	2	7	201	502	C	−	+	−	+	+	+	+	+
5	6	7	303	303	C	+	+	+	+	+	−	−	+
6	1	7	303	501	C	−	−	−	+	−	−	−	−
7	2	4	302	602	C	+	±	−	+	+	+	+	+
8	3	4	201	201	C	−	−	+	+	−	−	+	−
9	2	4	302	602	C	−	−	−	+	+	−	+	+
10	2	2	602	602	C	−	−	−	+	−	−	−	−
11	3	4	301	501	C	+	−	−	+	+	−	+	−
12	2	4	301	602	C	−	−	−	+	−	−	+	−
13	3	4	302	604	C	+	+	−	+	−	−	+	−
14	3	6	402	501	C	−	−	−	+	−	−	−	−
15	4	7	301	301	C	−	−	−	+	−	−	−	−
16	6	11	603	604	C	−	+	−	+	−	−	−	−
17	6	6	N.D.	N.D.	C	−	−	−	+	−	−	−	−
18	8	8	301	501	C	−	−	−	+	−	−	+	−
Total						4	6	2	18	8	3	10	4
% positive						22.2	33.3	11.1	100	44.4	16.7	55.6	22.2

only in tumors from patients 6 (DR4−) and 13 and 15 (DR4+). The disease status, tumor size, lymph node status, tumor grade, and HER-2 expression were also compared with the response to these peptides. There was no correlation between proliferative responses and these clinical parameters.

No apparent correlation was found between HER-2 overexpression and proliferative responses to HER-2 peptides. Of the three HER-2^{hi} patients (two DR4+ and one DR4−), responses to G89 were seen only in patient 13 (DR4+). These responses were stable on both day 4 and day 5 of testing. In contrast, patient 15 responded only to F13 (on day 6) (Fig. 1*E*), whereas patient 6 (DR4−) responded only to G88 and F7. Of the seven HER-2^{lo} patients, five showed responses to G89 on at least two consecutive days of assay, one showed a response on day 5 (S.I. >2.5) (Fig. 1*D*), and one failed to respond. This group was too small for statistical analysis to address possible differences in response due to HER-2 overexpression and disease progression.

Analysis of the significance and specificity of responses focused on characterizing the proliferative and cytokine response to G89 by T cells from patient 13 (HER-2^{hi}). To address the specificity of proliferative responses, G89-primed PBMCs from patient 13 (Fig. 2*A*) were expanded in culture with IL-2 and retested for their proliferative responses to G88, G89, and G90. Freshly isolated monocytes/macrophages were not available from this patient for restimulation and follow-up studies. We used PHA blasts from the same patient as APC. Results in Fig. 3*A* show that G89-stimulated cultures (G89R) recognized G89 significantly better than G88 and G90, but the overall level of response was low. In fact, no clear improvement in proliferative activity was seen after expansion in IL-2 without restimulation with peptide. Because this may be due to the poor APC ability of PHA blasts, the specificity of G89 stimulation was characterized in parallel with the response from donor 3 (also

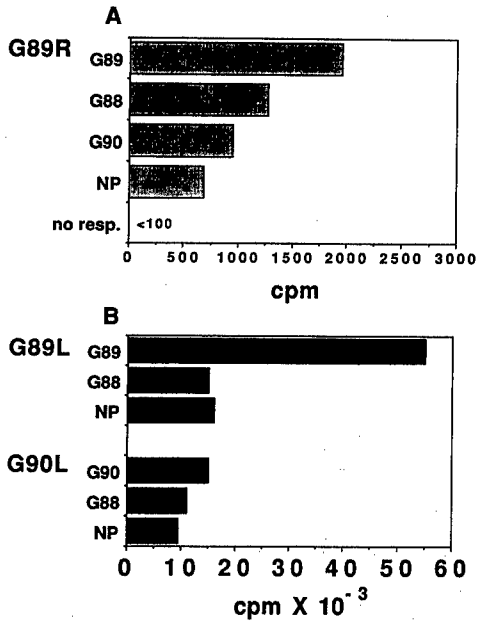


Fig. 3 *A*, specificity of proliferative responses of G89R T cells (derived after expansion in IL-2 of primary stimulated PBMC from patient 13). PHA blasts from patient 13 were used as autologous APC. *B*, specificity of proliferative responses of G89L and G90L (derived from donor 3). The G90L line was developed by priming with G90. Autologous plastic-adherent PBMCs were used as APC.

HLA-DR4+) because autologous APC, plastic-adherent cells were available. Cultured G89-primed PBMCs of this donor (designated G89L) showed significantly higher proliferative responses to G89 than to control G88 peptide at restimulation

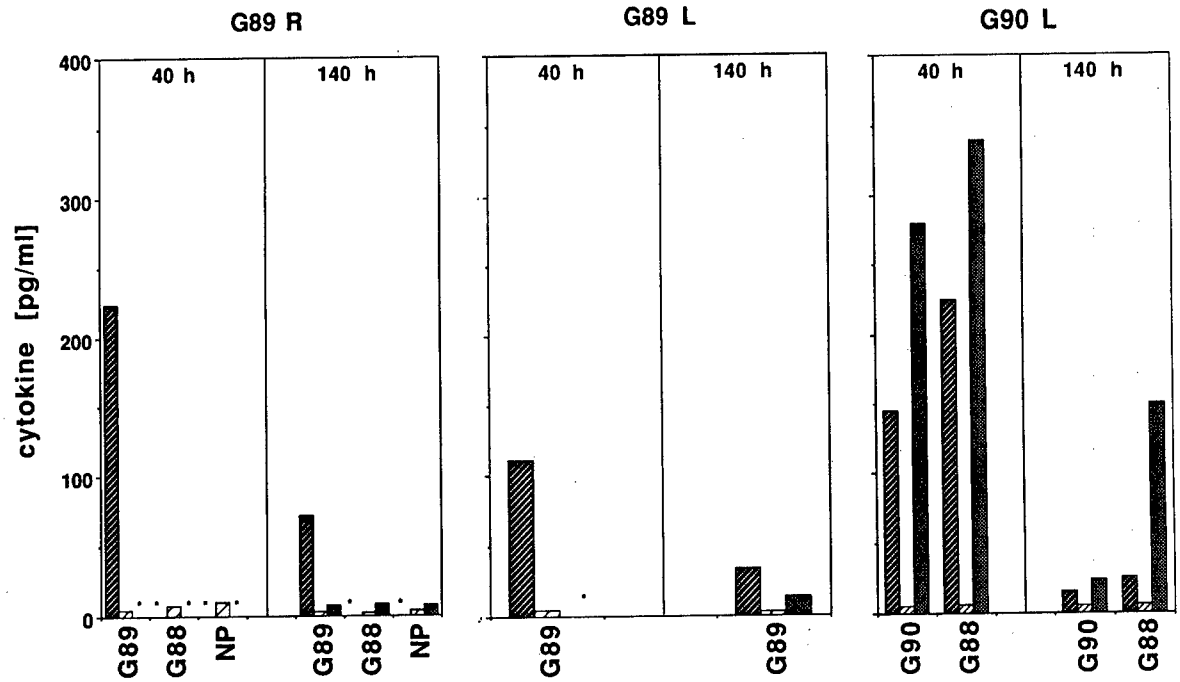


Fig. 4 Cytokine secretion by G89R and G90L T cells (5×10^4 cells each) in response to G89 and G90, respectively, pulsed on autologous irradiated PBMCs from donor 3 (1×10^5). G88 was used as control. Between 70 and 80% of cells had the CD4+ phenotype. Cytokine secretion by G89R and G90L was measured in the same experiment as described in "Materials and Methods." ▨, IFN-γ; ▨, IL-4; ▨, IL-10. *, levels of this cytokine were below the sensitivity of the assay (2 pg/ml).

when presented on autologous plastic-adherent fraction of PBMCs (Fig. 3*B*). In contrast, the corresponding G90-primed (G90L cells) showed significantly lower specific proliferation to recall with G90 than G89L cells to recall with G89, suggesting that G90 may prime the T cells for a cross-reactive epitope.

Secretion of IFN-γ by G89-stimulated T-Cell Lines. Because the levels of IFN-γ and IL-4 in primary cultures were either very low or undetectable (data not shown), studies were conducted on secondary cultures. Recent studies have shown that IL-2 is required for Th2 differentiation and IL-4 production (35). To determine the type of cytokine responses to G89, cultures were established in low-dose IL-2 after initial stimulation of PBMCs with G89 from patient 13 (G89R) and donor 3 (G89L) and with G90 from donor 3 (G90L). The ability of these cells to secrete IFN-γ, IL-4, and IL-10 in response to the priming peptide was tested in parallel with the control peptide G88.

In preliminary experiments, we observed that the levels of IL-4 and IL-10 in response to G89 were low or undetectable. Because secretion of IL-4 and IL-10 may be delayed or HER-2 peptide G89 may be less efficient in inducing Th2 cytokines, we first determined the cytokine profile in response to G89 and G90 at both 40 and 140 h. The peptide G88 was used as control. The G89R T cells secreted high levels of IFN-γ in response to G89 but not in response to control G88 peptide (Fig. 4). These cells also secreted significantly less IL-4 and IL-10 than IFN-γ after either 40 or 140 h in culture, suggesting a preferential Th1 or Th0-Th1 response to G89. However, because the responses determined were obtained with short-term bulk cultures and because background levels of IL-4 and IL-10 were present, we defined this reactivity as Th0-Th1. The G90-primed G90L T cells showed significant cross-reactivity with G88 with regard to IFN-γ and IL-10 secretion and secreted significantly more IL-10 than did G89R G89-induced T cells. Although the levels of IFN-γ in response to wither G90 or G88 were higher than the levels of IL-4, the levels of IL-10 were higher than the levels of IFN-γ. Although it is possible that earlier levels of IFN-γ secreted in response to G90 may have been higher, the very high levels of IL-10 may suggest the presence of nonspecific Th2 cells activated following the initial G90 stimulation.

To address whether IFN-γ was secreted in response to G89 and the ICD (which contains this peptide but not the control G88 peptide), in association with HLA-DR4, G89R and G89L T cells were tested for cytokine secretion in response to G89 presented by PBMCs of different phenotypes (Table 3). Comparison of MHC I phenotype between APC and responders suggested that the IFN-γ was not secreted in response to MHC I. G89L shared HLA-A2 and HLA-B44 with APC from donor B. Although this may raise the possibility that G89, G88, or shorter fragments can be presented by HLA-A2, APC from donor C also shared HLA-A2 with G89L and expressed HLA-23, B41, and 81. However, the levels of IFN-γ secreted were low compared with the levels detected when G89 was presented by APC from donors A and B. Thus, although the possibility that MHC I molecules may present G89 cannot be excluded, comparison of the MHC I phenotypes suggest that G89-stimulated T cells secrete cytokines in response to MHC II molecules.

Significantly higher levels of IFN-γ than IL-4 were se-

Table 3 MHC class II restriction of peptide and HER-2 protein recognition by G89-induced T-cell lines

			G89L[a] (DR4, 15, DQ6, 7)		G89R[a] (DR4, 3, DQ3, 6)	
	APC	Peptide	IFN-γ	IL-4	IFN-γ	IL-4
A	A2, B7, 44	G89	250	10.5	341	39.5
	DR4, 15; DQ6, 7	G88	26	11.1	73	25.0
		ICD	298	17.9	662	10.0
	APC only	G89	<2	<2	<2	<2
B	A1, 2, B44, 57	G89	155	17.8	125	10.4
	DR10, 15, DQ1, 6	G88	<2	<2	<2	<2
		ICD	<2	<2	<2	<2
	APC only	G89	<2	4.7	<2	<2
C	A2, 23, B41, 81	G89	18.2	<2	59	<2
	DR7, 11, DQ2, 6	G88	2.2	2.5	<2	<2
			<2	<2	<2	<2

[a] The G89L and G89R T cell lines were stimulated with 1 μM HER-2 peptides (G89, G88) or 1 μM HER-2 ICD in the presence of autologous APC, with G89L, *i.e.*, APC sharing all DR and DQ with G89L, and HLA-DR4 and HLA-DQ6 with G89R (A); APC sharing only DR15 and DQ6 with G89L, and only DQ6 with G89R (B); and APC sharing only HLA-DQ6 with G89L (C). Supernatants were collected after 40 h in culture. Cytokine levels were determined as described in "Materials and Methods."

creted by G89L cells in response to G89 and ICD when G89 was presented by autologous APC to G89L. A similar pattern of response was observed for the G89R cells obtained from the breast cancer patient. The IFN-γ response by both G89L and G89R to the control G88 peptide, although significant, was at least one order of magnitude lower than to G89, or to the ICD. In this experiment, APC (from donor 3) and responders shared only HLA-DR4 and HLA-DQ6. Thus, in the presence of APC that shared either (*a*) DR15 and DQ6 with G89L, (*b*) DQ6 with G89L, or (*c*) DQ6 with G89R, significant levels of IFN-γ were observed in response to G89 but not to ICD, by both responders, the healthy donor and the cancer patient. The results also show that the restriction element used by G89L and G89R for recognition of exogenously added peptides is not exclusively HLA-DR4. These results suggest that it is likely that a naturally processed peptide from HER-2 is recognized by HER-2 peptide G89-primed T cells in the context of HLA-DR4.

IFN-γ and IL-4 release was observed in response to G89 (but not to ICD) presented by APC sharing either HLA-DR15 or HLA-DQ6 or both with the responders. These levels of cytokines were not induced in response to G88. Although the levels of cytokines secreted when G89 was presented by other HLA molecules were lower than levels in response to HLA-DR4, a certain pattern of "promiscuous" recognition was present, consisting always of higher levels of IFN-γ than IL-4. This suggests that although MHC class molecules of DR4-APC could present exogenously loaded G89 in a form recognizable to G89L and G89R T-cell receptor, the naturally processed and presented fragment of the ICD may have been derived from the one presented by DR4. A similar pattern of responses by G89L and G89R, although with reduced IFN-γ levels, was seen using lymphoblastoid cell lines WT51 (homozygous for DR4) and E4181324 (homozygous for DR15) as APC (data not shown).

DISCUSSION

In this report, we present evidence that PBMCs from primary breast cancer patients respond by proliferation *in vitro* to a number of HER-2 peptides. The responding population consists of CD4+ cells, as demonstrated in a previous study (16) and as suggested by the ability of the responding cells to secrete IFN-γ in response to these peptides when presented by MHC class II. In previous experiments, we noted that anti-MHC II antibodies and, to a lesser extent, anti-MHC I antibodies inhibited proliferation of PBMCs to helper peptides (17). Low levels of proliferation compared with CD4+ cells were observed with isolated CD8+ cells in a healthy donor, but the differences in cpm between G89-primed (G89L) and G90-primed CD8+ cells from donor 3 were significant. However, given the length of these peptides, the stimulatory potential for CD8+ cells after the binding of G89 to certain HLA class I allele products deserves further investigation. The frequency of the responses was higher for G89 (56%) than for the other peptides tested, suggesting that G89 may represent an immunodominant epitope in the group analyzed. Of interest, the responses to G89 appeared to associate more frequently with the presence of HLA-DR4 (in seven of nine cases), suggesting that HLA-DR4 may be the presenting element.

The fact that F7 and G89 are equal in length and differ by one residue at their NH_2- and COOH-terminal ends suggests that the epitope formed by G89 *in vitro*, when used at a concentration of ~10 μM, is specifically recognized. The frequency of responses appeared not to be related to the binding affinity of these peptides to DR4. The predicted binding affinity of G89 to HLA-DR4 was significantly lower than that of peptides G88 and G89 of the same length.

It is unknown at this time whether for G89, binding to HLA-DR is sequence specific, is restricted to certain DR4 subtypes, or is promiscuous. Depending on which P1 frame is used, Tyr or Trp can serve as an anchor for DRB1*0401 but not for *0404 and *0402. Similarly, at P4, negatively charged residues Asp and Glu are accepted by DRB1*0401 and *0404 but not by *0402, which accepts positively charged residues, such as Lys/Arg (36). This suggests that G89 (as well as G90) may preferentially bind to different DR4 subtypes and use alternative binding frames (*i.e.*, with Val, Leu, and Met for the P1 frame). Additional studies are required to address the ques-

tion of whether the antigenicity of G89 is associated with the predicted poor binding in a fashion similar to that reported for most tumor peptides from self Ag recognized by human CTLs.

T-cell cultures primed with G89 responded at restimulation by secreting more IFN-γ than IL-4 and IL-10, suggesting the preferential activation of a Th1 response. Because the experiments were performed with bulk cultures and not with clones, and because IL-4 and IL-10 were detectable, we would rather define this reactivity as Th0-Th1. This response was apparently not directed to a cryptic HER-2 epitope because peptide-primed cells recognized the ICD. The IFN-γ response to ICD of G89-primed T cells suggests that HLA-DR4 may be the presenting element for a naturally processed epitope similar in structure to G89.

Recent studies to examine proliferative responses in breast and ovarian cancer using HER-2 peptides of various lengths and randomly selected patients who had not been HLA-typed showed T-cell responses to several HER-2 peptides (16, 17). One of those, defined as p783 (HER-2, 783–797), reportedly activated responses of T cells to the HER-2 protein in a breast cancer patient (16). Although the magnitude of G89-induced responses was significantly lower than that reported for p783, our results indicate a trend of increased proliferation to G89. F7 (HER-2, 776–788) was also found to induce T-cell proliferation in both healthy donors and ovarian cancer patients (17). The data in this study suggest that within the area HER-2, 776–797, nests a dominant HER-2 epitope for CD4+ cells. Because HLA-DR4 is expressed in approximately 25% of humans, this epitope may be an important peptide for activation and regulation of T-cell differentiation toward a Th1 response. It may also be beneficial for CTL activation and expansion.

The observation that T cells from both healthy donors and patients whose tumors overexpress HER-2 can respond to G89 argues against the induction of tolerance to this epitope and/or against autoimmune activation of G89-specific T cells by HER-2 only after protein overexpression. In both this and the previous study with p783 (16), the proliferative responses were observed early, 4–6 days after stimulation. This may argue against a primary response to G89. Primary *in vitro* responses of T cells to some foreign Ag have been shown to require a significantly longer time (7–9 days) to be detected as significant proliferation (37, 38), although it is unknown whether these findings can be extended to self Ag. The low levels of cytokines at primary stimulation may even argue against a recall response, unless the frequency of G89-specific cells is very low. Additional studies are needed to clarify this point. A possibility that needs to be considered is that epitopes such as G89 may induce *in vivo* a limited number of Th1 cells, which may exert a regulatory function. Preliminary studies in our laboratory show that primary stimulation of T cells from healthy donors with either F7 or F12 or F13 followed by culture in IL-2 leads to preferential expansion of F7-responsive cells. These cells secreted high levels of IFN-γ at secondary and tertiary stimulation with F7. This pattern of responses suggests a determinant spreading effect as described for some cryptic epitopes (39).[4]

[4] B. W. Anderson, A. P. Kudelka, M. Pollack, J. L. Murray, and C. G. Ioannides,, manuscript in preparation.

Previous studies of HER-2 focused primarily on characterizing CTL epitopes (40, 41). The observation, in different systems, that human tumors are antigenic although poorly immunogenic emphasizes the need for development of approaches to induce and augment an immune response to tumor. Although *in vitro* and *in vivo* models show that induction of tumor-specific CTLs can be achieved by costimulation (42), the observed activation of Th1 response by the same tumor Ag recognized by CTLs suggests an involvement of CD4+ cells in the reaction to tumor. It also raises the question of whether the G89-induced Th0-Th1 response plays a protective role during tumor spread or whether it is down-regulated by Th2 cytokines subsequent to recognition of other peptides after HER-2 overexpression. In this context, the characterization of epitopes that regulate Th1 responses, which can in turn control the spread of Th1/Th2 responses by other self peptides, may have important implications not only for CTL induction but also for understanding the regulation of human tumor immunity.

ACKNOWLEDGMENTS

We thank Susan Mondragon for expert editorial assistance.

REFERENCES

1. Cheever, M. A, Britzmann-Thompson, D., Klarnet, J. P., and Greenberg, P. D. Antigen-driven long-term cultured T cells proliferate *in vivo*, distribute widely, mediate specific tumor therapy and persist long-term as functional memory T cells. J. Exp. Med., *163:* 1100–1112, 1986.

2. Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J, Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H., and White, D. E. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. J. Natl. Cancer Inst., *86:* 1159–1166, 1994.

3. Boon, T., and van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. J. Exp. Med., *183:* 725–729, 1996.

4. Mitchell, M. S. Attempts to optimize active specific immunotherapy for melanoma. Int. Rev. Immunol., *7:* 331–347, 1991.

5. Topalian, S L. MHC class II restricted tumor antigens and the role of CD4+ T cells in cancer immunotherapy. Curr. Opin. Immunol., *6:* 741–745, 1994.

6. Topalian, S. L., Rivoltini, L., Mancini, M., Markus, N. R., Robbins, P. F., Kawakami, Y., and Rosenberg, S. A. Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. USA, *91:* 9461–9465, 1994.

7. Topalian, S. L., Gonzales, M. J., Parkhurst, M., Li, Y. F., Southwood, S., Sette, A., Rosenberg, S. A., and Robbins, P. F. Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted Tyrosinase epitopes. J. Exp. Med., *183:* 1965–1971, 1996.

8. Yoshino, I., Goedegebuure, P. S., Peoples, G. E., Lee, K-Y., and Eberlein, T. J Human tumor infiltrating CD4+ T cells react to B cell lines expressing heat shock protein 70. J. Immunol., *153:* 4149–4158, 1994.

9. Takahashi, T., Chapman, P. B., Yang, S. Y., Hara, I., Vijayasandhi, S., and Houghton, A. N. Reactivity of autologous CD4+ T lymphocytes against human melanoma. J. Immunol., *154:* 772–779, 1995.

10. Dadmarz, R., Sgagias, M K., Rosenberg, S A., and Schwartzentruber, D. J. CD4+ T lymphocytes infiltrating human breast cancer recognize autologous tumor in an MHC-class II restricted fashion. Cancer Immunol. Immunother., *40:* 1–9, 1995.

11. Heike, M., Schlaak, J., Schulze-Bergkamen, H., Heyl, S., Herr, W., Schmitt, U., Schneider, P. M., and Meyer zum Buschenfelde, K. H. Specificities and functions of CD4+ HLA- class II-restricted T cell clones against a human sarcoma: evidence for several recognized antigens. J. Immunol., *156:* 2205–2213, 1996.

12. Qin, H., Chen, W., Takahashi, M., Disis, M. L., Byrd, D. R., McCahill, L., Bertram, K. A., Fenton, R. G., Peace, D. J., and Cheever, M. A. CD4+ T-cell immunity to mutated ras protein in pancreatic and colon cancer patients. Cancer Res., *55:* 2984–2987, 1995.

13. Gjersen, M. K., Bakka, A., Breivik, J., Saeterdal, I., Solheim, B. G., Soreide, O., Thornsby, E., and Gaudernack, G. Vaccination with mutant ras peptides and induction of T-cell responsiveness in pancreatic carcinoma patients carrying the corresponding ras mutation. Lancet, *346:* 1399, 1995.

14. Takahashi, M., Chen, W., Byrd, D., Disis, M. L., Huseby, E., McCahill, L., Nelson, H., Peace, D., Qin, H. L., Shimada, H., Okuno, K., Yasutomi, M., and Cheever, M. A. Antibody responses to ras proteins in patients with colon cancer. Clin. Cancer Res., *1:* 1071–1077, 1995.

15. Tilkin, A. F., Lubin, R., Soussi, T., Lazar, V., Janin, N., Mathieu, M. C., Lefrere, I., Carlu, C., Roy, M., Kayibanda, M., Bellet, D., Guillet, J. G., and Bressac-dePaillerets, B. Primary proliferative T cell response to wild-type p53 protein in patients with breast cancer. Eur. J. Immunol., *25:* 1765–1769, 1995.

16. Disis, M. L., Calenoff, E., McLaughlin, G., Murphy, A. E., Chen, W., Groner, B., Jeschke, M., Lydon, N., McGlynn, E., Livingston, R. B., Moe, R., and Cheever, M. A. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res., *54:* 16–20, 1994.

17. Fisk, B., Hudson, J. M., Kavanagh, J., Wharton, J. T., Murray, J. L., Ioannides, C. G., and Kudelka, A. P. Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res., *17:* 45–54, 1997.

18. Lee, J. E., Lowy, A. M., Thompson, W. A., Lu, M., Bell, B., Skibber, J. M., Evans, D. B., Curley, S. A., Mansfield, P. F., and Reveille, J. D. Association of gastric adenocarcinoma with the human leukocyte antigen class II gene. DQB1*0301. Gastroenterology, *111:* 426–432, 1996.

19. Lee, J. E., Reveille, J. D., Ross, M. I., and Platsoucas, C. D. *HLA-DQB1*0301* association with increased melanoma risk. Int. J. Cancer, *59:* 510–513, 1994.

20. Lee, J. E., Lu, M., Mansfield, P. F., Platsoucas, C. D., Reveille, J. D., and Ross, M. I. Malignant melanoma: relationship of the human leukocyte antigen class II gene *DQB1*0301* to disease recurrence in American Joint Committee on Cancer stage I or II. Cancer (Phila.), *78:* 758–763, 1996.

21. Lee, J. E., Loflin, P. T., Laud, P. R., Lu, M., Reveille, J. D., and Lawlor, D. A. The human leukocyte antigen *TAP2* gene defines the centromeric limit of melanoma susceptibility on chromosome 6p. Tissue Antigens, *47:* 117–121, 1996.

22. Marsh, S. G. E., and Bodmer, J. G. HLA class II region nucleotide sequences. Tissue Antigens, *45:* 258–280, 1995.

23. Kropshofer, H., Max, H., Muller, C. A., Hesse, F., Stevanovic, S., Jung, G., and Kalbacher, H. Self-peptide released from class II HLA-DR1 exhibits a hydrophobic two-residue contact motif. J. Exp. Med., *175:* 1799–1803, 1992.

24. Malcherek, G., Falk, K., Rotzschke, O., Rammensee, H-G., Stevanovic, S., Gnau, V., Jung, G., and Melms, A. Natural peptide ligand motifs of two HLA molecules associated with myasthenia gravis. Int. Immunol., *5:* 1229–1237, 1993.

25. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H-G. Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. Immunogenetics, *39:* 230–242, 1994.

26. Wucherpfennig, K. W., and Strominger, J. L. Selective binding of self peptides to disease-associated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases. J. Exp. Med., *181:* 1597–1601, 1995.

27. Hammer, J., Valsasnini, P., Tolba, K., Bolin, D., Higelin, J., Takacs, B., and Sinigaglia, F. Promiscuous and allele-specific anchors in HLA-DR binding peptides. Cell, *74:* 197–203, 1993.

28. Sinigaglia, F., and Hammer, J. Motifs and supermotifs for MHC class II binding peptides. J. Exp. Med., *181:* 449–451, 1995.

29. Marshall, K. W., Wilson, K. J., Liang, J. Woods, A., Zaller, D., and Rothbard, J. B. Prediction of peptide affinity to HLA-DRB1*0401. J. Immunol., *154:* 5927–5933, 1995.

30. Ioannides, C. G., Fisk, B., Fan, D., Biddison, W. A., Wharton, J. T., and O'Brian, C. A. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptides derived from the HER-2/neu proto-oncogene. Cell. Immunol., *151:* 225–234, 1993.

31. Ioannides, C. G., and Freedman, R. S. T cell responses to ovarian tumor vaccines. Int. Rev. Immunol., *7:* 349–364, 1991.

32. Fleiss, J. L. Statistical Methods for Rates and Proportions, pp. 83–85. John Wiley & Sons, New York, 1973.

33. Sheen-Chen, S-M., Chou, F-F., Eng, H-L., and Chen, W-J. An evaluation of the prognostic significance of HLA-DR expression in axillary-node-negative breast cancer. Surgery (St. Louis), *116:* 510–515, 1994.

34. Casoli, C., Zanelli, P., Adorni, A., Starcich, B. R., and Neri, T. Serological and molecular study on the HLA phenotype of female breast cancer patients (Letter). Eur. J. Cancer, *30:* 1207–1208, 1994.

35. Kamogawa, Y., Minasi, L. A., Carding, S. R., Bottomly, K., and Flavell, R. A. The relationship of IL-4- and IFN-γ-producing T cells studies by lineage ablation of IL-4 producing cells. Cell, *75:* 985–995, 1993.

36. Hammer, J., Gallazzi, F., Bono, E., Karr, R. W., Guenot, J., Valsasnini, P., Nagy, Z. A., and Sinigaglia, F. Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association. J. Exp. Med., *181:* 1847–1855, 1995.

37. Barker, R. N., and Elson, C. J. Multiple self epitopes on the Rhesus polypeptides stimulate immunologically ignorant human T cells. Eur. J. Immunol., *24:* 1578–1582, 1994.

38. Elson, C. J., Barker, R. N., Thompson, S. J., and Williams, N. A. Immunologically ignorant autoreactive T cells, epitope spreading and repertoire limitation. Immunol. Today, *16:* 71–76, 1995.

39. Nanda, N. K., and Sercarz, E. E. Induction of anti-self-immunity to cure cancer. Cell, *82:* 13–17, 1995.

40. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of HER-2/neu-proto-oncogene recognized by ovarian tumor specific CTL lines. J. Exp. Med., *181:* 2709–2717, 1995.

41. Peoples, G. E., Goedegebuure, P. S., Smith, R., Linehan, D. C., Yoshino, I., and Eberlein, T. J. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER-2/neu-derived peptide. Proc. Natl. Acad. Sci. USA, *92:* 432–436, 1995.

42. Allison, J. P., Hurwitz, A. A., and Leach, D. R. Manipulation of costimulatory signals to enhance antitumor T-cell responses. Curr. Opin. Immunol., *7:* 682–686, 1995.

Cancer Immunol Immunother (1999) 48:401–410

ORIGINAL ARTICLE

Larry D. Anderson Jr. · J. Michael Hudson
Cherylyn A. Savary · Bryan Fisk · David M. Gershenson
Constantin G. Ioannides

HER-2/*neu* peptide specificity in the recognition of HLA-A2 by natural killer cells

Received: 16 March 1999 / Accepted: 3 June 1999

Abstract Although natural killer (NK) cells have been described as non-MHC-restricted, new evidence suggests that NK activity can be either up- or down-regulated after interaction with the peptide–MHC-class-I complex expressed on target cells. However, the epitope(s) recognized by NK cells have remained ill-defined. We investigated NK cell recognition of synthetic peptides representing a portion of a self-protein encoded by the HER-2/*neu* (HER-2) proto-oncogene and presented by HLA-A2. HER-2 nonapeptides C85, E89, and E75 were found partially to protect T2 targets from lysis by freshly isolated and interleukin-2(IL-2)-activated NK cells (either HLA-$A2^+$ or $A2^-$). This inhibition was not solely due to changes in the level of HLA-A2 expression or conformation of serological HLA-A2 epitopes. Using single-amino-acid variants at position 1 (P1) of two HER-2 peptides, we observed that protection of targets was dependent on the sequence and the side-chain. These results suggest similarities in the mechanism of target recognition by NK and T cells. This information may be important for understanding the mechanisms of tumor escape from immunosurveillance and could help explain the aggressiveness of HER-2-overexpressing tumor cells.

Key words Natural killer cells · HER-2/*neu* · Peptides · MHC · Tumor immunity

L.D. Anderson Jr. · J.M. Hudson · B. Fisk
D.M. Gershenson · C.G. Ioannides (✉)
The University of Texas M.D. Anderson Cancer Center,
Department of Gynecologic Oncology,
Box 67, 1515 Holcombe Blvd., Houston, Texas 77030, USA
e-mail: ioannides.constantin@gynonc.mdacc.tmc.edu
Tel.: +1-713-792-2849
Fax: +1-713-792-7586

C.A. Savary
The University of Texas M.D. Anderson Cancer Center,
Department of Surgical Oncology,
Box 18, 1515 Holcombe Blvd., Houston, Texas 77030, USA

Introduction

Natural killer (NK) cells are thought to play an important role in the elimination of virus-infected cells and cancer cells [5, 26, 39]. Although target-cell killing by NK cells has traditionally been described as non-MHC-restricted, interaction of NK-cell-inhibitory receptors with MHC class I molecules often leads to a down-regulation of NK cytolytic function in proportion to the level of MHC class I expression on the targets [20, 27, 40, 42]. Recent reports also indicated that single amino acid mutations within the peptide-binding groove of the MHC molecule can affect target cell sensitivity to lysis, suggesting that NK cells recognize different conformations induced by peptides bound in the MHC class I pockets [19, 37]. This hypothesis has been supported by observations that external loading of target cells with either self or foreign peptides can enhance or inhibit sensitivity to NK-mediated lysis in a peptide-specific manner independent of the level of MHC class I up-regulation [7, 25, 28, 38]. However, the basis for peptide specificity in the induction of lysis or protection is unknown. Further analysis of the mechanism of NK recognition of peptides may provide an important insight into the function of NK cell specificity for tumor cells.

The HER-2/*neu* (HER-2) proto-oncogene product is overexpressed in a variety of human cancers including breast, ovarian, colon, lung, and stomach, and its overexpression by breast and ovarian cancers has been shown to correlate with earlier relapse and a worse prognosis [36]. Since it has been reported that HER-2 overexpression also correlates with decreased NK cell activity [41], we wanted to determine if HER-2 peptides are directly involved in NK cell inhibition. Therefore, we used HER-2 peptides recognized by cytotoxic T lymphocytes (CTL) as targets, where the question of the sequence specificity in NK recognition can be addressed. In this report we investigated the ability of freshly isolated and in vitro interleukin-2(IL-2)-activated NK cells to recognize self-HER-2 peptides that bind to the HLA-

A2 molecule with variable affinities and induce conformational changes in the α1 and α2 domains. We found that these peptides decreased NK-mediated lysis of T2 cells, and the ability to inhibit lysis depended more upon peptide sequence than the ability to up-regulate or induce conformational changes of HLA-A2 on the target cells. Interestingly, the peptide that induced the most HLA-A2 up-regulation and conformational changes inhibited lysis least. Furthermore, targets pulsed with HER-2 peptide variants containing amino acid substitutions at position 1 (P1) showed either side-chain-dependent protection or increased sensitivity to NK-mediated lysis. Again, increased levels of lysis inhibition among the peptides did not correlate with increased levels of expression of HLA-A2, as detected by the W6/32 mAb specific for a monomorphic MHC I epitope (α3 domain), and lysis inhibition did not correlate with conformational changes of HLA-A2 detected by the MA2.1 mAb (α1 domain). Peptides that induced the most change in expression and conformation of HLA-A2 were often less effective at inhibiting lysis. However, the enhanced sensitivity to NK lysis seen with one peptide was paralleled by changes in the conformational epitope recognized by the BB7.2 mAb (α2 domain). These results indicate an important effect of changes in peptide sequence at position 1, and provide further evidence that the mechanism of NK target recognition has some similarity to that of T cells in that it is determined by interactions of peptide side-chains with NK receptors. These findings may also be helpful in explaining why cancer patients with tumors overexpressing HER-2 have a worse prognosis.

Materials and methods

Target cells

The T2 line has been described previously [15] and was a generous gift from Dr. Peter Cresswell (Yale Univeristy School of Medicine, New Haven, Conn.). The B cell line C1R:A2, an HLA-A2-gene-transfected derivative of C1R, was a gift from Dr. William Biddision (National Institute of Neurological Disorders, Bethesda, Md.). C1R:A2 cells were transfected with the plasmid pCMV.HER-2 encoding a full-length HER-2 cDNA (the kind gift of Dr. Mien-Chie Hung, Department of Tumor Biology, M.D. Anderson Cancer Center). C1R:A2:HER-2 transfectants were selected by resistance to hygromycin B by co-transfection of SV2.Hygro plasmids (ATCC, Rockville, Md.).

Effector cells

Peripheral blood buffy coats of normal donors were purchased from a local blood center, and mononuclear cells (PBMC) prepared by Ficoll-Hypaque gradient separation [24]. NK cells were enriched to high purity by negative selection using a MACS NK Isolation Kit (Miltenyi Biotec, Auburn, Calilf.). In brief, PBMC were incubated for 15 min at 4 °C with a cocktail of monoclonal antibodies (mAb) recognizing CD3, CD4, CD19, and CD33, washed, and then incubated for an additional 15 min with colloidal superparamagnetic microbead-labelled antibody reacting to the primary antibodies (Beckton Dickinson, Mountain View, Calif.). The cells were then passed twice through an iron-wool column placed within a strong magnetic field, and the nonadherent cells collected. The effluent population was routinely 91.7%–98.2% $CD56^+$, $CD3^-$ NK cells, 0.1%–1.4% $CD56^+$, $CD3^+$ T cells, and 0.2%–1.3% $CD56^-$, $CD3^+$ T cells as determined by two-color flow cytometry [35].

For IL-2 activation, NK cells were cultured for 5–7 days in RPMI-1640 medium supplemented with 10 mM HEPES buffer, 10% human AB serum, antibiotics, 2 mM glutamine, 2 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol (complete RPMI medium), and 500 U/ml highly purified human recombinant rIL-2 (18×10^6 IU/mg; Cetus Corp., Emeryville, Calif.). The NK cell line, NKL (kindly provided by Dr. M.J. Robertson, Dana Farber Cancer Institute, Boston, Mass.) was obtained from peripheral blood of a patient with a $CD3^-$, $CD16^+$, $CD56^+$ large granular lymphoproliferative disorder [18]. These cells were maintained in culture in complete RPMI medium supplemented with 30 U/ml IL-2.

In some experiments, the CTL line 41 (CTL-41) was used as a source of effectors. This line was developed by repeated in vitro stimulation of $HLA\text{-}A2^+$ peripheral blood mononuclear cells from a healthy donor with peptide C84: HER-2 (971–979 V) and a longer peptide C43: HER-2 (968–981) [12]. For these studies, CTL-41 were maintained in culture with monthly restimulation with 10 μg/ml C84 peptide and autologous or allogeneic $HLA\text{-}A2^+$ PBMC. The CTL used as effectors were selected on mAb-coated plates (AIS Micro CELLector, Applied Immune Sciences, Menlo Park, Calif.), and were $CD3^+$, $CD4^-$, $CD8^+$. Clones were isolated from the CTL-41 line, as previously described [14].

Synthetic peptides

Synthetic peptides corresponding to sequences in HER-2: E75 (369–377), E89 (851–859), C85 (971–979), and recognized by ovarian tumor-specific CTL, have been reported previously [12–14]. The amino acid sequences of these peptides are shown in Table 1. Variants of the C85 peptide substituted at P1 are designated as G1, F1, T1, and K1 [12]. The E75 peptide substituted at P is designated as peptide F41. The synthetic peptides used in this study were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, purified to 92%–95% by HPLC, and dissolved in phosphate-buffered saline (PBS) at a stock concentration of 1 mg/ml.

Table 1 Sequences of synthetic HER-2 peptides

Code	Position	1	2	3	4	5	6	7	8	9
E75	369–377	K	I	F	G	S	L	A	F	L
F41		G	–	–	–	–	–	–	–	–
E89	851–859	V	L	V	K	S	P	N	H	V
C85	971–979	E	L	V	S	E	F	S	R	M
G1		G	–	–	–	–	–	–	–	–
F1		F	–	–	–	–	–	–	–	–
T1		T	–	–	–	–	–	–	–	–
K1		K	–	–	–	–	–	–	–	–
C84		E	–	–	–	–	–	–	–	U

Cytotoxicity assay

The ^{51}Cr release assay has been described in detail previously [24]. For peptide-pulsing experiments, ^{51}Cr-labelled T2 cells were dispensed into 96-well microtiter plates and preincubated for 2 h in serum-free RPMI medium, to which was added either 10 µl peptide (100 µg/ml final concentration), or an equivalent volume of PBS as a control. Effector cells, suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, were then added in various E:T ratios (ranging from 40:1 to 1:1), and the culture supernatants were tested for chromium release after 4–5 h of culture. Each experimental condition was tested in triplicate. Results are expressed as the percentage specific lysis according to the formula $(E - S)/(M - S) \times 100$, where E is the radioactivity (cpm) of experimental wells containing both effectors and targets, S is the spontaneous release of ^{51}Cr from targets incubated in medium (with and without peptide), and M represents the radioactivity for targets incubated with 0.2% Triton X-100 (maximum release). In some experiments the cytotoxicity was expressed as lytic units (LU), where 1 LU is the number of effector cells required for lysis of 30% of the target cells [35]; when this calculation is used, the results are expressed as $LU_{30}/10^6$ effector cells.

In studies designed to analyze the sensitivity of HER-2-gene-transfected cell lines to lysis, NK or CTL effector cells were incubated for 4–5 h with ^{51}Cr-labelled C1R:A2:HER-2-transfected target cells at effector-to-target ratios (E:T) ranging from 12:1 to 50:1

Flow-cytometric analysis

Expression of HLA-A2 on T2 target cells was evaluated by flow cytometry, using BB7.2, MA2.1 and W6/32 mAb. W6/32 mAb (Dako, Dakopatts, Denmark) recognizes a monomorphic epitope common to HLA-A, -B, and -C. The anti-HLA-A2 mAb, BB7.2 (mouse IgG2b) and MA2.1 (mouse IgG1) were obtained from the American Type Culture Collection (ATCC). Other antibodies used in this study included anti-CD11a, anti-CD18, anti-CD58, and anti-CD56 (Beckton-Dickinson, Mountain View, Calif.); and Ab2, reacting with the extracellular domain of HER-2 protein (Oncogene Science, Uniondale, New York). Briefly, 5×10^5 cells were incubated for 30 min at 4 °C with primary antibody (or an isotype control antibody nonreactive with human cells), washed, and then incubated for an additional 30 min with fluorescein-isothiocyanate-conjugated goat anti-(mouse Ig). Flow-cytometric analyses were performed on 5000 gated events/sample, using a FACScan flow cytometer (Becton-Dickinson, Mountain View, Calif.) and Consort 30 software.

To analyze the effect of peptide pulsing on HLA-A2 expression, T2 cells were incubated for 2 h at 37 °C with 10–100 µg/ml peptide (or PBS alone as a control), prior to labelling with the primary mAb. All cells tested were positive for HLA-A2 expression; data are reported as the mean channel fluorescence, indicative of the channel number corresponding to the average peak of fluorescence [6, 31, 35].

Statistical analysis

The data were analyzed statistically using Prism 2.01 software (GraphPad Prism for Scientists, Sorrento, Calif.). Multiple groups were compared by the Newman Keuls one-way analysis of variance. When only two groups were compared, Student's *t*-test was used. Differences were considered significant when P was less than 0.05.

Results

HER-2 peptides inhibit NK-mediated lysis of T2 cells

In the first series of experiments, we investigated the effects of HER-2 self-peptides on the sensitivity of T2 target cells to lysis by NK cells. The T2 cells have a defect in TAP (transporter-associated with antigen presentation) proteins and display "empty" HLA-A2 molecules [33] that can be loaded exogenously with peptides having the proper anchors for binding to HLA-A2, i.e. L/M/I/V (P2) and V/L/M/I (P9). For our studies we used three different synthetic nonapeptides of HER-2 that display these HLA-A2 anchors: E75, E89 and C85 (the amino acid sequences of these peptides are shown in Table 1). These peptides were previously found to reconstitute recognition of $CD8^+$, $CD4^-$ CTL lines derived from ovarian tumor-associated lymphocytes [12], suggesting that HER-2 is naturally processed into identical or similar peptides presented by HLA-A2 on tumor cells.

The T2 targets were pulsed with peptides at a concentration of 100 µg/ml prior to addition of effector cells. In agreement with others [32], we observed that untreated T2 targets were sensitive to lysis by freshly isolated and IL-2-activated peripheral blood NK cells from all healthy donors tested (Fig. 1). However, T2 cells pulsed with HER-2 peptides were significantly protected from killing by unstimulated HLA-$A2^+$ and HLA-$A2^-$ NK cells (Fig. 1A, B). These peptides also protected T2 targets from lysis by IL-2-activated HLA-$A2^+$ and HLA-$A2^-$ NK cells (Fig. 1C, D). The results from four representative donors of eight tested are shown in Fig. 1. The reduction in lysis of peptide-pulsed

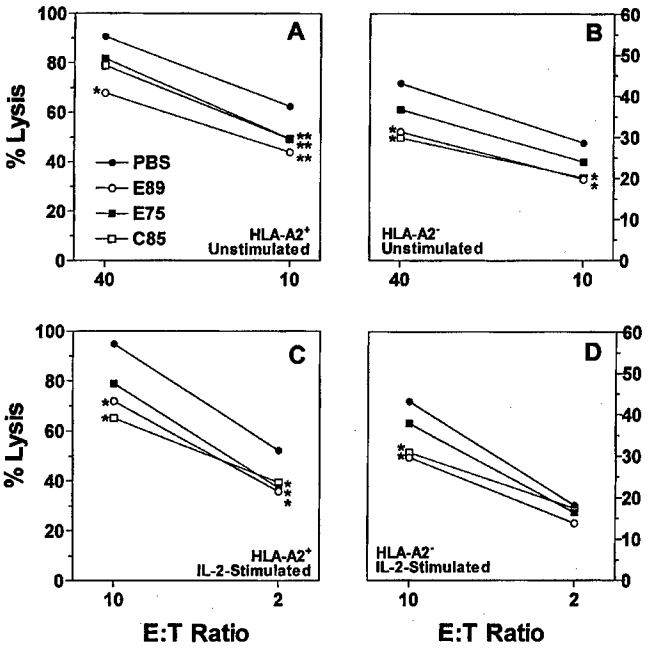


Fig. 1A–D Recognition of HER-2 peptide-pulsed T2 cells by natural killer (NK) cells. Magnetically sorted NK cells from four healthy donors (one per panel) were tested for lytic activity against HER-2-peptide-pulsed T2 cells in a ^{51}Cr-release assay. The NK cells were (**A**) unstimulated HLA-$A2^+$, (**B**) unstimulated HLA-$A2^-$, (**C**) interleukin-2(*IL-2*)-stimulated HLA-$A2^+$, and (**D**) IL-2-stimulated HLA-$A2^-$. Significant inhibition of lysis: $^*P < 0.05$ compared to phosphate-buffered saline (*PBS*) control; $^{**}P < 0.01$ compared to PBS control

target cells was consistently observed at multiple E:T ratios and ranged from 15% to 30%.

Because sensitivity of target cells to NK-mediated lysis has been shown to be inversely related to the levels of MHC class I expression [40], we next determined whether the resistance of HER-2-pulsed T2 targets to lysis by NK cells was associated with an increase in MHC class I molecules caused by peptide-induced stabilization [6]. As shown in Fig. 2, T2 cells incubated with HER-2 peptides displayed an increase in the relative density of surface HLA class I molecules as detected by the HLA-A, -B, -C-specific W6/32 mAb; this was observed as an increase in the fluorescence intensity of mAb-labelled peptide-pulsed T2 cells compared to controls. However, there were marked differences among the peptides in the relative density of class I molecules induced, with an approximately twofold and threefold increase caused by E89 and E75 respectively. The C85 peptide only slightly increased MHC class I expression. Despite these large differences in MHC class I expression, the level of protection afforded by E89 and C85 peptides was comparable. Although E75 increased MHC expression the most, it was consistently least effective at inhibiting target lysis (Fig. 1). These results indicate that the increased resistance of T2 to lysis by NK cells induced by C85 was not simply caused by up-regulation of MHC class I molecules.

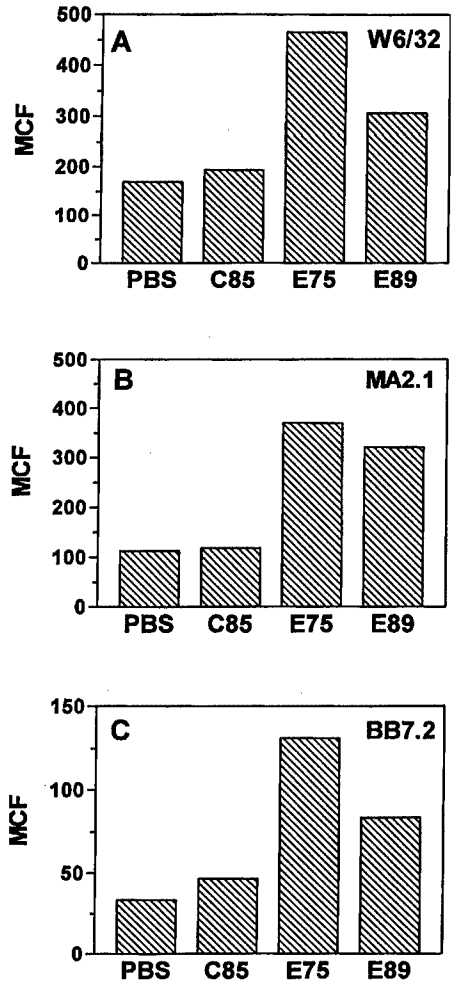


Fig. 2A–C Up-regulation of HLA-A2 expression by HER-2 peptides. T2 cells pulsed with or without (PBS control) HER-2 peptides, were analyzed by flow cytometry for expression of epitopes recognized by the W6/32, MA2.1, and BB7.2 mAb. *Bars* the mean channel fluorescence (*MCF*) value, i.e., the channel corresponding to the mean fluorescence intensity of positively stained cells

To determine if peptide-induced protection was related to changes in the conformation of HLA-A2 molecules, we also analyzed peptide-pulsed and control T2 cells for expression of conformational epitopes recognized by BB7.2 and MA2.1 mAb. The epitope recognized by the BB7.2 mAb is located on the N-terminal loop of the α2 domain (including W108) of HLA-A2, in an area not expected to contact the peptide directly [34]. MA2.1 mAb reacts with the α1 domain of HLA-A2 at residues 64–68, which border the A and B pockets of the peptide-binding groove; mutations of HLA-A2 in this area have been reported to affect T cell recognition significantly [16, 34]. The results in Fig. 2 indicate that the decrease in the sensitivity of T2 to lysis by NK cells, after pulsing with a particular peptide, was not proportional to the increase in the expression of either of these conformational epitopes. On the contrary, while MA2.1 and BB7.2 epitopes were expressed at approximately threefold higher levels on E75-pulsed T2 cells compared to untreated or C85-pulsed targets, the protection induced by E75 was, in most cases, less than that of the other peptides (Fig. 1).

Recognition of peptide variants by NK cells

Crystallography studies have shown that the N-terminal (P1) residue of peptides binds within the A pocket of the MHC molecule, and that the nature of the side-chain of this residue affects peptide binding to HLA-A2 [3]. To address the question of whether a single amino acid substitution at P1 would alter the ability of a HER-2 peptide to protect targets from NK-mediated lysis, we created a series of C85 variants by replacing the glutanic acid at P1 with lysine (variant K1), glycine (variant G1), threonine (variant T1) or phenylalanine (variant F1) (Table 1). These peptide variants do not have changes in the dominant anchors for HLA-A2 at P2 (L/M/I/V) and P9 (V/L/M/I), so they should still bind to HLA-A2. Using a peptide concentration that was protective for C85, we tested the ability of the peptide variants to protect T2 cells from NK-mediated lysis (Fig. 3A). Significant differences between the peptides were observed regarding their ability to affect T2 lysis. Specifically, we found that the K1 variant was as effective as the natural C85 peptide in protecting T2 cells from lysis, while the F1 and G1 variants did not significantly inhibit T2 lysis compared to control T2 cells treated with PBS. In contrast, T2 targets pulsed with the T1 variant not

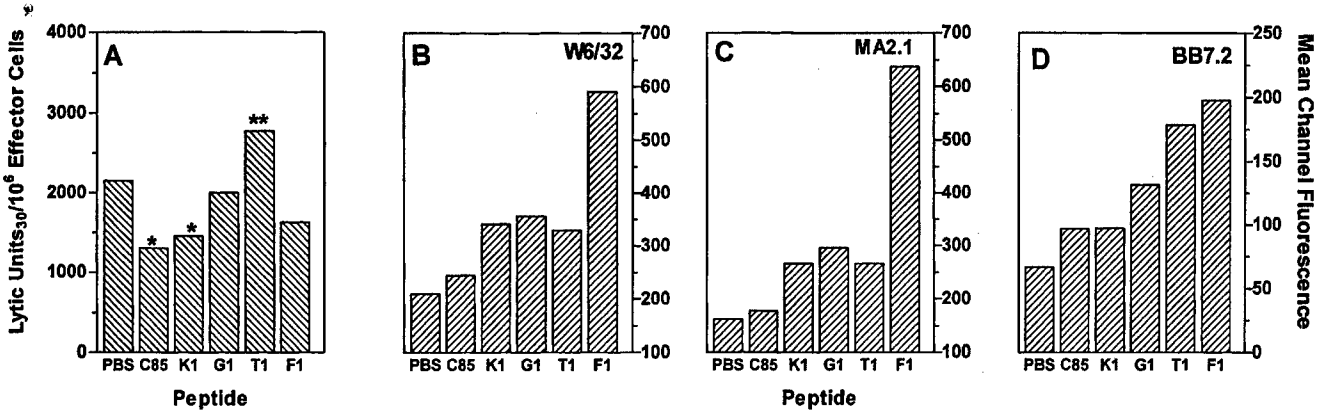


Fig. 3A–D Recognition of HER-2 peptide variants by NK cells. **A** Immunomagnetically isolated HLA-A2$^+$ NK cells were tested for cytolytic activity against T2 targets pulsed with 100 μg/ml C85 and C85 variants, and the results from four separate donors were averaged. Data are expressed as lytic units as described in Materials and methods. * There was significant protection of T2 after pulsing with C85 (E1) or the K1 variant ($P < 0.05$ compared to PBS-treated control T2 targets). ** The T1 variant caused significant enhancement of T2 lysis compared to both C85-pulsed and control targets ($P < 0.05$). **B–D** Three different mAb (W6/32, MA2.1, and BB7.2) were used to detect MHC class I expression by T2 cells pulsed with the same peptides used in **A**

only were not protected, but instead were even more susceptible to lysis by NK cells than were PBS-treated controls. On the basis of a comparison of cytotoxicity (LU), they were also twofold more susceptible to NK lysis than C85-pulsed T2.

All of these C85 variants up-regulated and stabilized MHC class I expression as detected by the W6/32 mAb, albeit to different degrees (Fig. 3B). The conformational epitopes recognized by MA2.1 and BB7.2 mAb were also up-regulated when compared to PBS-treated T2 targets (Fig. 3C, D). The levels of expression of W6/32 and MA2.1 HLA-A2 epitopes on T2 cells pulsed with K1, G1, and T1 variants were similar, and they were higher than the levels induced by C85. The F1 variant induced a twofold higher increase in these epitopes relative to the other variants. The T1 and F1 variants induced the highest levels of BB7.2 epitope expression among the variants tested.

When the levels of expression of MHC class I and the BB7.2 and MA2.1 conformational epitopes were compared to protection from lysis, the ability of a particular peptide to down-regulate target sensitivity to NK lysis was not directly proportional to the increase in the level of HLA-A2 expression. For example, neither the G1 nor F1 variant was significantly protective, even though F1 induced a substantially higher expression of W6/32, MA2.1, and BB7.1 epitopes. Furthermore, compared to C85, the T1 peptide enhanced the susceptibility of T2 cells to NK-mediated lysis, even though this variant induced higher levels of HLA-A2 expression and conformational changes. The K1 variant was as protective as C85, but induced higher levels of MHC class I than did the natural peptide, as detected by the W6/32 and MA2.1 mAb. Again, these data support the observation that increased MHC class I does not always correlate with enhanced resistance to lysis by NK cells.

The results in Fig. 3 show a significant role for the P1 residue side-chain in NK inhibition. Protective peptides in this study (C85 and K1) have charged side-chains, while the nonprotective peptides (F1 and G1) have nonpolar side-chains. T1, which enhanced NK sensitivity, has a hydroxyl group. To confirm that the NK inhibition is dependent on the side-chain of the amino acid at P1, we investigated NK recognition of T2 cells pulsed with the weakly NK protective peptide E75 and its P1 variant, F41. In the latter variant, the lysine at P1 in the natural peptide is substituted with glycine, which lacks a side-chain (Table 1). This K → G change at P1 of E75 is identical to the change between the K1 and G1 variants of C85 described above. Thus F41 was expected to be less protective than E75. We observed that both E75 and F41 showed a similar concentration-dependent ability to up-regulate HLA-A2 expression on T2 cells: for example, at 100 μg/ml, the mean channel fluorescence for the expression of the BB7.2 epitope was approximately threefold higher for both E75-pulsed and F41-pulsed targets than for the controls (data not shown). Both peptides also protected T2 targets from lysis by the NK cell line (NKL) when used at concentrations of 10–100 μg/ml (Fig. 4A). However, at 200 μg/ml, E75 (charged P1 side chain) was significantly more protective ($P < 0.001$) against NK lysis than was F41 (nonpolar P1 side-chain), supporting the conclusion from the previous experiment.

To rule out the possibility that the HER-2 peptides were stabilizing non-HLA-A2 MHC or nonclassical MHC molecules that could mediate a decrease in NK sensitivity [1, 2], E75-pulsed T2 targets were treated with either the HLA-A2-specific MA2.1 mAb or control antibody before lysis by the NK cell line was assessed. As seen in Fig. 4B, HER-2-peptide-induced inhibition of lysis was most likely mediated directly through interaction with HLA-A2, because MA2.1 antibody completely blocked the inhibition of T2 lysis at 100 μg/ml E75 ($P < 0.05$) and significantly blocked the higher inhibition at 200 μg/ml E75 ($P < 0.01$).

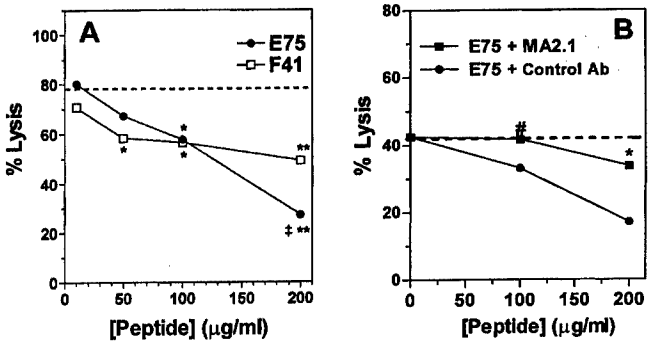


Fig. 4A, B Protection of T2 targets by HER-2 peptides is concentration-dependent and HLA-A2-dependent. T2 cells pulsed with E75 or its variant, F41, were tested for susceptibility to lysis by the HLA-A2$^+$ NK cell line, NKL, at an E:T ratio of 40:1. - - - - The percentage lysis of PBS-treated control T2 targets (78% for **A** and 42% for **B**). **A** Significant protection from lysis: * $P < 0.01$ compared to PBS-treated control; ** $P < 0.001$ compared to PBS control; ‡ E75 inhibited lysis significantly better than F41 at 200 μg/ml ($P < 0.001$). **B** E75-pulsed T2 targets were incubated with HLA-A2-specific blocking antibody (MA2.1) or control antibody. # MA2.1 completely blocked the protection from lysis at 100 μg/ml E75 ($P < 0.05$). * Significant blocking of the protection from lysis also occurred at 200 μg/ml E75 ($P < 0.01$)

Recognition of HER-2-transfected C1R:A2 cells by NK cells

Under physiological conditions, NK cells interact with HLA-A2 molecules presenting peptides processed endogenously. Therefore, it was of interest to determine if endogenously processed HER-2 peptides could also protect target cells from lysis by NK cells. We approached this question using C1R:A2 cells transfected with the HER-2 gene (C1R:A2:HER-2$^+$ cells). C1R:A2:HER-2$^+$ cells were cloned by stringent limiting dilution, and three clones (HER-2:G, HER-2:B, and HER-2:L), expressing different levels of surface HER-2, were tested for sensitivity to lysis by CTL and NK cells. CTL clones (CD3$^+$, CD8$^+$, CD4$^-$) were developed by in vitro stimulation of HLA-A2$^+$ mononuclear cells from a healthy donor with C84, a P9-substituted (M → V) C85 peptide [12]. As shown in Fig. 5, two clones (2C2 and 2C3) recognized the C1R:A2:HER-2:L clone but not C1R:A2 cells lacking HER-2 gene expression, suggesting that an epitope similar to C85 was presented by C1R:A2:HER-2$^+$ cells. However, the HER-2$^+$ clones were more resistant to lysis by IL-2-stimulated NK cells than were the nontransfected

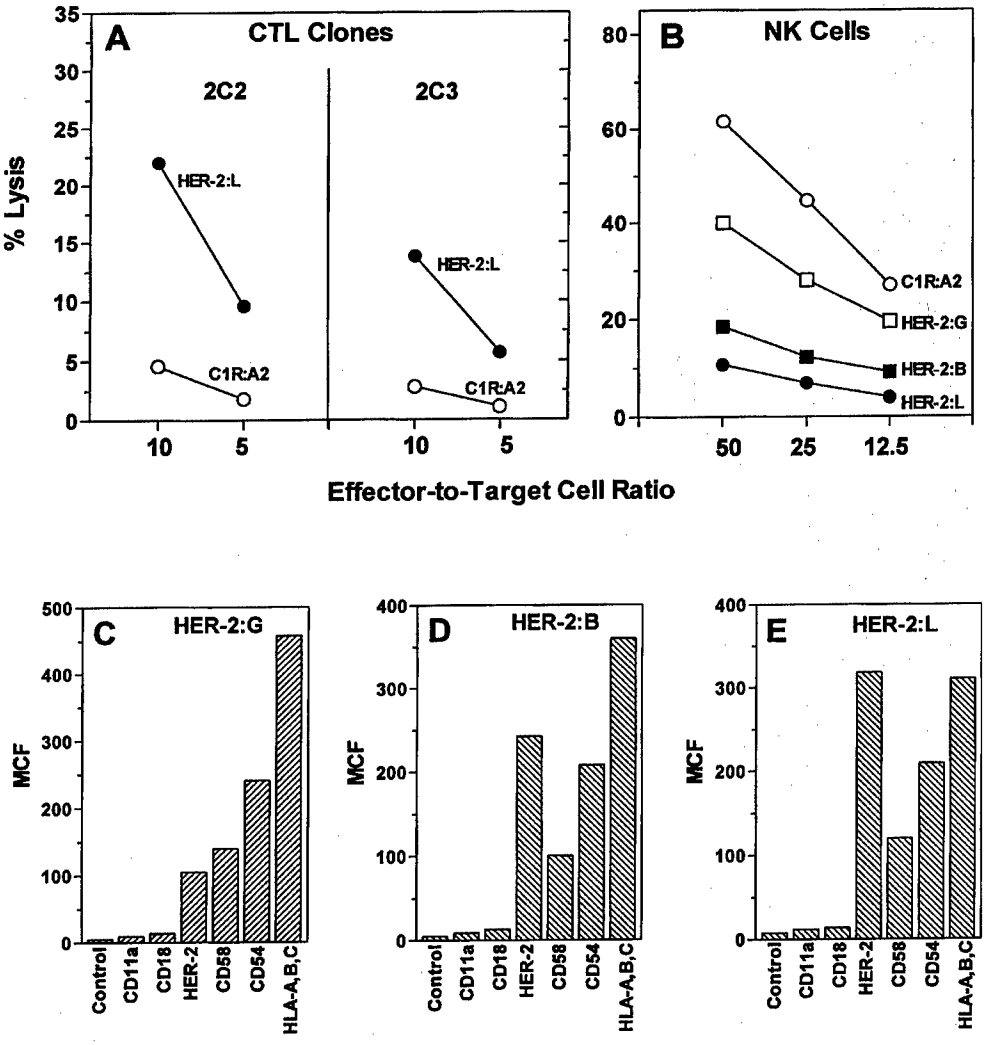


Fig. 5A–E Lysis of the HER-2 gene-transfected target cells by cytotoxic T lymphocytes (CTL) and NK cells. **A, B** Nontransfected and HER-2-transfected CIR:A2 cells (HER-2:G, HER-2:B, and HER-2:L) were tested for susceptibility to lysis by two C85-specific HLA-A2$^+$ CTL clones (2C2 and 2C3) and magnetically sorted, IL-2-activated HLA-A2$^+$ NK cells. The HER-2 transfectants were clones chosen for their high (HER-2:L), medium (HER-2:B), or low (HER-2:G) expression of HER-2. **C, D, E** Surface MHC class I, HER-2, LFA-1a (CD11a), LFA-1b (CD18), LFA-3 (CD58), and ICAM-I (CD54) expression by C1R:A2 and HER-2 transfectants was analyzed by flow cytometry

targets. Furthermore, the sensitivity of the clones to lysis by NK cells varied inversely with the density of HER-2 expression; i.e. the sensitivity of the transfectants to lysis ranked G > B > L, while HER-2 expression ranked L > B > G (Fig. 5).

The HER-2:G targets were most sensitive to NK lysis even though they expressed a higher density of HLA-A2 than did HER-2:B and HER-2:L. Additional phenotypic analyses of these clones revealed that they all expressed only very low levels of CD18 (LFA-1β) and CD11a (LFA-1α), while CD54 (ICAM-1) and CD58 (LFA-3) were expressed at similar levels among the cloned transfectants (Fig. 5C–E). Therefore, there was no correlation between adhesion molecule expression and the sensitivity of HER-2 transfectants to lysis by NK cells. Our data suggest that quantitative and qualititative changes in the composition of the naturally processed HER-2 peptides presented by MHC, rather than alterations in the expression of MHC class I or adhesion molecules, are responsible for the protective effects of HER-2.

Discussion

In this report we present novel evidence that HLA-A2-binding HER-2 peptides, known to form CTL epitopes, can protect targets from lysis by NK cells. This protection was found to be dependent upon (a) peptide concentration, requiring pulsing with peptides at 50–100 μg/ml; (b) peptide sequence, since single amino-acid substitutions could significantly alter the status of target susceptibility; and (c) side-chain charge, with charged side-chains at position 1 generally inducing more protection from NK lysis than uncharged side-chains. In support of previous studies by others [2, 25, 26, 28, 43], this indicates that NK cells recognizing peptide-MHC complexes display a high degree of target specificity. These findings also suggest that CTL epitopes on tumor cells may block NK lysis, a mechanism that may have implications for tumor survival in the absence of CTL. An increase in the relative ability of a peptide to inhibit lysis was, in most cases, not associated with increased expression of HLA-A2 on T2 target cells, or with conformational changes of HLA-A2 detected by BB7.2 and MA2.1, suggesting that these serological epitopes are not solely responsible for inhibition of NK function.

HLA-A2 conformational changes were often seen on targets that were most sensitive to lysis in this study. For example, increased staining with the BB7.2 mAb was associated with enhanced lysis in the case of the T1 peptide and decreased protection from lysis for E75. One possible explanation for the enhanced sensitivity to lysis of targets bearing HLA-A2 conformational changes could be that, although HLA-A2 expression inhibits lysis, it can only do so if the conformation is not altered by the peptide. However, the full explanation is probably more complex, because E89 induced a fair amount of HLA-A2 conformational changes (both MA2.1 and BB7.2) yet inhibited lysis as effectively as C85, a peptide that did not induce such changes in HLA-A2. One alternative explanation for the enhanced sensitivity to lysis caused by T1 is the hydroxylated side-chain (tyrosine) at P1, which may have decreased the recognition of HLA-A2–peptide by an inhibitory NK receptor. Further experiments are necessary to elucidate this mechanism.

In agreement with previous studies, the peptide concentrations required to induce a significant NK-protective effect were higher than the concentrations required to sensitize T2 cells to CTL effectors from breast and ovarian cancer patients [12, 14]. This may indicate that these effects are only relevant in vitro. However, recent studies on peptide binding to HLA-A2 molecules indicate that, during 4–6 h of incubation, the number of class I MHC complexes formed with similar amounts of exogenously added peptides is in the range of 10^3–10^4, which is consistent with the level of expression of a number of endogenous peptides [17]. Therefore our results should be relevant to certain pathological conditions, such as viral infections and cancer, where large amounts of viral or tumor peptides are processed and presented by MHC class I. The observation that NK cells were less effective in lysis of C1R:A2 cells expressing high levels of HER-2, than of those expressing lower levels, is suggestive of this possibility. Thus, protection from NK-mediated lysis may be dependent not only on the presence of self-peptides or MHC, but also on the high-density expression of specific peptide-MHC complexes. These findings are compatible with the use of an NK-inhibitory receptor with low affinity for the recognition of peptide-MHC complexes. Furthermore, the same peptides were capable of inhibiting lysis of HLA-$A2^+$ T2 cells by NK effectors from both HLA-$A2^+$ and HLA-$A2^-$ donors, indicating that the receptor(s) responsible for this inhibition are expressed independently of HLA-A2 expression in the donors.

These studies were performed using highly enriched (up to 98% purity) NK cells, to exclude a role for T cells in any of the observed effects. We also observed that HER-2 peptides protected targets from lysis by an established NK cell line. In no experiment, though, was complete protection of T2 cells by HER-2 peptides observed. This is not surprising, because the NK cells used in our studies were not clones. It has been shown that different NK clones can respond differently to the same peptide-pulsed targets [8, 9, 23, 25], most likely because of expression of different combinations of inhibitory and activation receptors. Bulk NK populations were used in most of our experiments to mimic more closely the effector/tumor conditions existing in vivo. In fact, it is important to realize that the 15%–30% of tumor cells that might be protected from NK cells by HER-2 peptides would represent a substantial number of malignant cells likely to escape NK cell attack.

Our results show that a side-chain charge at P1 of two different HER-2 peptides is important for protection from lysis. It is interest that the requirement for a specific side-chain in the protection of a target against NK-mediated lysis suggests that certain NK receptors, or

structures on NK cells involved in target lysis, directly contact MHC-bound peptide. Importantly, these effects were observed for the first time when peptides known to induce CTL-mediated lysis in the HLA-A2 system were used. Recent studies have shown sequence-specific NK-potentiating effects for P8 of nonapeptides, although the effects were not associated solely with charged residues at P8 [26]. Furthermore, Peruzzi and collaborators identified a role for P7 and P8 of HLA-B*2705-associated peptides in modulation of NK recognition [28]. Charged side-chains in residues at P7 and P8 in their system enhanced NK-mediated lysis. These studies indicate that residues in certain positions of the class-I-MHC-bound peptides can up- or down-modulate NK lysis. Nevertheless, the effects may be dependent upon HLA type or other unknown factors, which may help explain why one donor of four tested (Fig. 3) in our study showed a somewhat different-from-average pattern of NK inhibition by the C85 variants (inhibition by F1 and G1 but not K1; data not shown). It is most likely that HER-2 peptides were inhibiting lysis directly through the interaction of HLA-A2–peptide complexes with NK receptors, since A2-specific mAb significantly blocked the inhibition. Although the inhibition was not completely blocked when high levels of peptide were used, likely explanations are that monomorphic HLA-A2 was up-regulated more than the MA2.1 conformational epitope or that the antibody was not saturating the HLA-A2 at high peptide concentrations. This could also possibly be due to peptide stabilization of non-classical MHC, such as the deletion variants described by Abu-hadid et al. [1].

Positive stimulation (activation) of NK cells may occur through several different activation or costimulatory receptors on NK cells, such as NKR-P1 proteins, CD16 and CD28, but it appears that the specificity of NK target recognition is often not provided by activation signals, but rather by the presence or absence of inhibitory signals induced by recognition of peptide-MHC complexes [20]. It has been suggested that peptide-induced protection from NK cells may be due to stabilization and/or conformational effects of peptides on MHC class I molecules. However, the role of the peptide in NK recognition is probably not simply to stabilize MHC class I or to promote changes in MHC conformation. NK cells express an array of different receptors that inhibit target cell lysis upon recognition of MHC class I. Examples are the C-type lectin superfamily of receptors (e.g. CD94, NKG2) and the killer-cell-inhibitory receptors of the immunoglobulin superfamily (e.g. p 70, p 58) [4, 20, 29]. Several investigators have now demonstrated that inhibitory receptors on NK cells not only recognize specific types of MHC but also recognize a specific subset of peptides on HLA-B or C [4, 25, 28–30, 43]. Our results in the HLA-A2 system also show that NK cell recognition is sensitive to mutations in peptides that minimally affect monomorphic MHC class I expression. Furthermore, changes in the expression of conformational MHC epitopes did not appear to cause the inhibition of NK-mediated lysis in this model, although such epitopes may have caused increased sensitivity to lysis, as discussed above. It is tempting to hypothesize that NK receptors use a similar mechanism of recognition to the one recently proposed for the T cell receptor [10]; i.e., the proper conformation of the MHC-peptide complex is required for the receptor to "land" on the target, while the changes in side-chain moieties (charge, polarity, van der Waals forces), are responsible for initiation of signaling. This will explain why expression of the MA2.1 conformational epitope does not correlate with recognition, since the epitope recognized by MA2.1 mAb is directly affected by side-chains of residues in pocket A (and possibly B) of HLA-A2, while the BB7.2 mAb detects altered conformation induced by the peptide in a different position (α2 domain, W108), which does not interact directly with peptide side-chains. More extensive studies are needed to address this point, but this study suggests that a number of mutations in peptides (including CTL epitopes) presented by MHC class I may interfere with MHC recognition by NK cells. These findings may have implications for understanding the mechanism by which cells infected with viruses (e.g. influenza or AIDS), and displaying a high rate of mutation, might escape immune defenses. This mechanism may also apply to tumor cells where overexpression of certain gene products (e.g. tyrosinase, gp100, or Muc-1) could lead to the presentation of a high density of self-epitopes with inhibitory effect on NK cells. An additional possibility to be examined is that presentation of mutated peptides (e.g. from p53 or p21 *ras*) may protect tumor cells from NK surveillance.

In support of our conclusions, it has been shown previously that HER-2-overexpressing breast and ovarian cell lines were more resistant to NK-mediated lysis than nonexpressing (or HER-2^{low}) targets [21]. As was the case also in our investigations, resistance in the latter studies could not be attributed solely to an increase in MHC class I or to changes in ICAM-1 expression by the HER-2^{+} targets [11, 22]. Taken together, these results suggest that endogenously processed HER-2 peptides expressed in complexes with MHC class I molecules may contribute to the resistance of HER-2-overexpressing tumor cells to NK-mediated lysis. Therefore, further elucidation of how NK cells recognize peptides may help to explain the aggressiveness of some tumors, as well as provide new insight into the nature of NK cell receptors for antigens.

Acknowledgements The authors would like to thank Timothy W. King for his assistance in the preparation of this manuscript. Peptide synthesis was supported in part by core grant 16672. Supported in part by grants DAMD: 4313 and 7098.

References

1. Abu-hadid MM, Fuji H, Sood AK (1994) Alternatively spliced MHC class I mRNAs show specific deletion of sequences encoding the extracellular polymorphic domains. Int Immunol 6:323

2. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG (1998) Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J Exp Med 187:813
3. Bouvier M, Wiley DC (1994) Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. Science 265:398
4. Brooks AG, Borrego F, Posch PE, Patamawenu A, Scorzelli CJ, Ulbrecht M, Weiss EH, Coligan JE (1999) Specific recognition of HLA-E, but not classical, HLA class I molecules by soluble CD94/NKG2 A and NK cells. J Immunol 162:305
5. Brutkiewicz RR, Welsh RM (1995) Major histocompatibility complex class I antigens and the control of viral infections by natural killer cells. J Virol 69:3967
6. Catipovic B, Dal Porto J, Mage M, Johansen TE, Schneck JP (1992) Major histocompatibility complex conformational epitopes are peptide specific. J Exp Med 176:1611
7. Chadwick BS, Sambhara SR, Sasakura Y, Miller RG (1992) Effect of class I MHC binding peptides on recognition by natural killer cells. J Immunol 149:3150
8. Ciccone E, Pende D, Nanni L, Di Donato C, Viale O, Beretta A, Vitale M, Sivori S, Moretta A, Moretta L (1995) General role of HLA class I molecules in the protection of target cells from lysis by natural killer cells: evidence that the free heavy chains of class I molecules are not sufficient to mediate the protective effect. Int Immunol 7:393
9. Correa I, Raulet DH (1995) Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells. Immunity 2:61
10. Ding YH, Smith KJ, Garboczi DN, Utz U, Biddison WE, Wiley DC (1998) Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. Immunity 8:403
11. Fady C, Gardner AM, Gera JF, Lichtenstein A (1992) Interferon-induced increase in sensitivity of ovarian cancer targets to lysis by lymphokine-activated killer cells: selective effects on HER2/*neu*-overexpressing cells. Cancer Res 52:764
12. Fisk B, Chesak B, Pollack MS, Wharton JT, Ioannides CG (1994) Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/*neu* proto-oncogene in vitro. Cell Immunol 157:415
13. Fisk B, Chesak B, Wharton JT, Ioannides CG (1994) Sequence motifs of human HER-2 proto-oncogene important for peptide binding to HLA-A2. Int J Oncol 5:51
14. Fisk B, Blevins TL, Wharton JT, Ioannides CG (1995) Identification of an immunodominant peptide of HER-2/*neu* protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 181:2109
15. Henderson RA, Michel H, Sakaguchi K, Shabanowitz J, Appella E, Hunt DF, Engelhard VH (1992) HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. Science 255:1264
16. Hogan KT, Clayberger C, Bernhard EJ, Walk SF, Ridge JP, Parham P, Krensky AM, Engelhard VH (1989) A panel of unique HLA-A2 mutant molecules define epitopes recognized by HLA-A2-specific antibodies and cytotoxic T lymphocytes. J Immunol 142:2097
17. Kageyama S, Tsomides TJ, Sykulev Y, Eisen HN (1995) Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. J Immunol 154:567
18. Kaufman DS, Schoon RA, Robertson MJ, Leibson PJ (1995) Inhibition of selective signaling events in natural killer cells recognizing major histocompatibility complex class I. Proc Natl Acad Sci USA 92:6484
19. Kurago ZB, Smith KD, Lutz CT (1995) NK cell recognition of MHC class I. NK cells are sensitive to peptide-binding groove and surface alpha-helical mutations that affect T cells. J Immunol 154:2631
20. Lanier LL (1998) NK cell receptors. Annu Rev Immunol 16:359
21. Lichtenstein A, Berenson J, Gera JF, Waldburger K, Martinez-Maza O, Berek JS (1990) Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-activated killer cells: correlation with expression of HER2/*neu* oncogenes. Cancer Res 50:7364
22. Lichtenstein A, Fady C, Gera JF, Gardner A, Chazin VR, Kelley D, Berenson J (1992) Effects of beta-2 microglobulin anti-sense oligonucleotides on sensitivity of HER2/*neu* oncogene-expressing and nonexpressing target cells to lymphocyte-mediated lysis. Cell Immunol 141:219
23. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL (1994) NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. J Exp Med 180:537
24. Lotzova E, Savary CA, Herberman RB (1987) Induction of NK cell activity against fresh human leukemia in culture with interleukin 2. J Immunol 138:2718
25. Malnati MS, Peruzzi M, Parker KC, Biddison WE, Ciccone, E, Moretta A, Long EO (1995) Peptide specificity in the recognition of MHC class I by natural killer cell clones. Science 267:1016
26. Mandelboim O, Wilson SB, Vales-Gomez M, Reyburn HT, Strominger JL (1997) Self and viral peptides can initiate lysis by autologous natural killer cells. Proc Natl Acad Sci USA 94:4604
27. Moretta L, Ciccone E, Mingari MC, Biassoni R, Moretta A (1994) Human natural killer cells: origin, clonality, specificity, and receptors. Adv Immunol 55:341
28. Peruzzi M, Parker KC, Long EO, Malnati MS (1996) Peptide sequence requirements for the recognition of HLA-B*2705 by specific natural killer cells. J Immunol 157:3350
29. Peruzzi M, Wagtmann N, Long EO (1996) A p70 killer cell inhibitory receptor specific for several HLA-B allotypes discriminates among peptides bound to HLA-B*2705. J Exp Med 184:1585
30. Rajagopalan S, Long EO (1997) The direct binding of a p58 killer cell inhibitory receptor to human histocompatibility leukocyte antigen (HLA)-Cw4 exhibits peptide selectivity. J Exp Med 185:1523
31. Rohren EM, McCormick DJ, Pease LR (1994) Peptide-induced conformational changes in class I molecules. Direct detection by flow cytometry. J Immunol 152:5337
32. Salcedo M, Momburg F, Hammerling GJ, Ljunggren HG (1994) Resistance to natural killer cell lysis conferred by TAP1/2 genes in human antigen-processing mutant cells. J Immunol 152:1702
33. Salter RD, Cresswell P (1986) Impaired assembly and transport of HLA-A and -B antigens in a mutant T × B cell hybrid. EMBO J 5:943
34. Santos-Aguado J, Barbosa JA, Biro PA, Strominger JL (1988) Molecular characterization of serologic recognition sites in the human HLA-A2 molecule. J Immunol 141:2811
35. Savary CA, Lotzova E (1992) Adhesion molecules on MHC-nonrestricted lymphocytes: high density expression and role in oncolysis. Lymphokine Cytokine Res 11:149
36. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al. (1989) Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 244:707
37. Storkus WJ, Salter RD, Alexander J, Ward FE, Ruiz RE, Cresswell P, Dawson JR (1991) Class I-induced resistance to natural killing: identification of nonpermissive residues in HLA-A2. Proc Natl Acad Sci USA 88:5989
38. Storkus WJ, Salter RD, Cresswell P, Dawson JR (1992) Peptide-induced modulation of target cell sensitivity to natural killing. J Immunol 149:1185
39. Trinchieri G (1989) Biology of natural killer cells. Adv Immunol 47:187
40. Trinchieri G (1994) Recognition of major histocompatibility complex class I antigens by natural killer cells. J Exp Med 180:417

41. Wiltschke C, Tyl E, Speiser P, Steininger A, Zeillinger R, Kury F, Czerwenka K, Kubista E, Preis P, Krainer M, et al. (1994) Increased natural killer cell activity correlates with low or negative expression of the HER-2/*neu* oncogene in patients with breast cancer. Cancer 73:135
42. Yokoyama WM (1995) Natural killer cell receptors specific for major histocompatibility complex class I molecules. Proc Natl Acad Sci USA 92:3081
43. Zappacosta F, Borrego F, Brooks AG, Parker KC, Coligán, JE (1997) Peptides isolated from HLA-Cw*0304 confer different degrees of protection from natural killer cell-mediated lysis. Proc Natl Acad Sci USA 94:6313

JOURNAL OF INTERFERON AND CYTOKINE RESEARCH 22:583–592 (2002)

Axillary Lymph Node Cellular Immune Response to HER-2/*neu* Peptides in Patients with Carcinoma of the Breast

HENRY M. KUERER,[1] GEORGE E. PEOPLES,[1,7] AYSEGUL A. SAHIN,[2] JAMES L. MURRAY,[3,4] S. EVA SINGLETARY,[1] AGAPITO CASTILLEJA,[5] KELLY K. HUNT,[1] DAVID M. GERSHENSON,[5] and CONSTANTIN G. IOANNIDES[5,6]

ABSTRACT

HER-2/*neu* peptides have recently been shown to induce a proliferative response by peripheral CD4$^+$ T cells in breast cancer patients. To investigate potential differences in the local cellular immune response between breast cancer patients with and without nodal metastases, lymphocytes were isolated from axillary lymph nodes from patients with breast cancer, and proliferative and cytokine responses to HER-2/*neu* peptides were determined. Freshly isolated lymphocytes from lymph nodes of 7 women undergoing surgery for invasive breast cancer were plated at 20×10^5 cells per well in triplicate. Cells were stimulated with HER-2/*neu* peptides at 50 μg/ml and with control antigens. Incorporation of tritium-labeled thymidine was determined 4 days later. The levels of the cytokines interferon-γ (IFN-γ), interleukin-4 (IL-4), and IL-10 were determined at priming and at restimulation with HER-2/*neu* peptides using a cytokine-specific, double-sandwich, enzyme-linked immunosorbent assay (ELISA). Lymphocytes isolated from the axillary lymph nodes of the patients mounted significant cellular immune response to HER-2/*neu* peptides, manifested by proliferation and specific cytokine elaboration. Proliferative responses to HER-2/*neu* peptides were seen in lymphocytes of patients with and without overexpression of HER-2/*neu* in the primary tumor. In some patients, the proliferative response to HER-2/*neu* peptides in lymphocytes from lymph nodes with metastases was absent or blunted compared with the response in lymphocytes from lymph nodes without metastases from the same patient ($p < 0.05$). HER-2/*neu* peptides induced a predominantly T helper type 1 (Th1) pattern of cytokine response in nodal lymphocytes isolated from breast cancer patients. A Th1-specific cytokine production pattern was maintained at priming and restimulation with HER-2/*neu* peptides and was amplified with IL-12 costimulation. These results indicate that HER-2/*neu* peptides can activate T cells in draining lymph nodes from women with invasive breast cancer. This activation is associated with a predominantly Th1 cytokine response, which suggests that conditioning with HER-2/*neu* peptides may be of value in the development of breast cancer vaccines.

INTRODUCTION

THE HUMAN HER-2/*neu* GENE encodes a 185,000-kDa transmembrane tyrosine kinase growth factor receptor.[1] Overexpression of HER-2/*neu* occurs in up to one third of invasive breast cancers and is associated with aggressive disease, resistance to certain types of cytotoxic chemotherapy, and poor prognosis.[2–4] Overexpression of HER-2/*neu* is also known to occur in 50%–80% of cases of high-grade ductal carcinoma *in situ*.[2,5,6] Eradication of ductal carcinoma *in situ* may be an early means of preventing the potential development of invasive breast carcinoma.

The HER-2/*neu* protein appears to be an excellent target for breast cancer vaccine development because it is expressed at very low levels in normal tissues but is overexpressed in aggressive primary invasive breast carcinomas and in breast cancer metastases.[7] The use of anti-HER-2/*neu* monoclonal antibodies (mAb) has been shown in recent clinical trials to be effective in the treatment of women with HER-2/*neu*-overexpressing metastatic breast cancer.[8] Therefore, HER-2/*neu* vac-

Departments of [1]Surgical Oncology, [2]Pathology, [3]Breast Medical Oncology, [4]Bioimmunotherapy, [5]Gynecologic Oncology, and [6]Immunology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.
[7]Present address: Department of Surgery, Uniformed Services University of Health Sciences, Bethesda, MD 20307.

cines might prove valuable in the development of immunity to breast cancer and in boosting the immune response in patients with established HER-2/*neu*-overexpressing tumors.

In breast cancer patients, the draining axillary lymph nodes are exposed to tumor-related cellular products and are often the first detectable site of metastases. Thus, the axillary lymph nodes represent an excellent and unique model in which to study the local immune response in breast cancer patients.

Our laboratory has developed and tested several HER-2/*neu* peptides in an effort to better understand the mechanisms of tumor recognition by T lymphocytes and immunologic tolerance to tumor, to characterize the structure of tumor antigens, and to potentially develop peptide-based vaccines.[9–11] We have shown previously that these HER-2/*neu* peptides may induce proliferative responses in peripheral blood T lymphocytes in some breast and ovarian cancer patients.[12] In the present investigation, we have developed a novel human *ex vivo* model to investigate the local immune response in breast cancer patients. This study was designed to directly evaluate the local cellular immune response to HER-2/*neu* peptides in axillary lymph nodes from women with invasive carcinoma of the breast by measuring the proliferative and cytokine responses of lymphocytes isolated from lymph nodes with metastases and from those without metastases.

PATIENTS AND METHODS

Patients

Figure 1 illustrates the general experimental design of the current investigation. The University of Texas M. D. Anderson Cancer Center Institutional Review Board approved the study, and patients participating in the study gave informed consent to participate.

*Determination of HER-2/*neu *status*

The HER-2/*neu* status of the primary tumor was determined by immunohistochemical staining of 4-μm thick sections cut from a representative paraffin-embedded block of invasive carcinoma. The slides were incubated with the anti-HER-2/*neu* mAb e2-4001 (1:100 dilution) on a Dako autostainer (Dako Corp., Carpinteria, CA) with the LSAB-2 peroxidase kit (Dako) using 3-3-diaminobenzedine. The percentage of cells with complete membrane staining and the intensity of the staining were evaluated on a semiquantitative scale of 0–3+, with scores defined as follows: 3+, strong complete membrane staining in more than 10% of tumor cells; 2+, weak to moderate complete membrane staining in more than 10% of tumor cells; 1+, faint or barely perceptible membrane staining in more than 10% of tumor cells. In all cases exhibiting any positivity (1+–3+), we confirmed the immunoreactivity by performing fluorescence *in situ* hybridization analysis to evaluate the Her-2/*neu* gene copy number. We used the Path Vysion HER-2/*neu* kit (Vysis, Downers Grove, IL), which employs two directly labeled fluorescent DNA probes. One is specific for the HER-2/*neu* gene locus, and the second is specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17. Signals are counted for 40 tumor cells by using an epifluorescence microscope, and the ratio of HER-2/*neu* to chromosome 17 is calculated. A ratio >2.0 is considered to represent HER-2/*neu* gene amplification and is considered a positive test result.

*HER-2/*neu *peptides*

The HER-2/*neu* peptides were prepared by the Synthetic Antigen Laboratory at M. D. Anderson Cancer Center using a solid-phase method as previously described.[13,14] Peptides were synthesized by following the common motifs for all MHC class II molecules defined by pool sequencing of naturally processed peptides.[15] HER-2/*neu* peptide purity was 93%–97% as determined by high-performance liquid chromotography (HPLC). Peptides were dissolved in phosphate-buffered saline (PBS), aliquoted at 2 mg/ml, and stored frozen at −20°C until used.

Isolation, stimulation, and propagation of lymphocytes

Standard axillary dissection was performed, and the axillary contents were immediately examined grossly by a pathologist under a sterile hood located just outside the operating rooms. Lymph nodes were bivalved and microscopically examined for the presence of breast cancer axillary metastases using frozen section or touch preparation techniques or both. Half of each lymph node was submitted for permanent section examination to confirm the presence or absence of metastases. The other half of each lymph node was immediately mechanically dispersed and then plated at a concentration of 20×10^5 cells per well with RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) with 5% human serum and 40 μg/ml gentamicin in triplicate.

One hour after lymph node cellular dispersion, HER-2/*neu* peptides or PBS alone was added to each well at a final concentration of 50 μg/ml. When no proliferation was evident on peptide stimulation, interleukin-12 (IL-12) (PharMingen, San Diego, CA) was added as a costimulator at a concentration of 200 ρg/ml. IL-12 has demonstrated significant antitumor activity in several preclinical tumor models.[16,17] Therefore, it was of interest to investigate the effects of IL-12 in the current breast cancer model. For restimulation experiments, after 4 days of stimulation with HER-2/*neu* peptides, the cultures were expanded with IL-2 (Cetus Corporation, Emeryville, CA) at 20 U/ml for 1 week. The cultures were maintained for 3 days in the absence of IL-2 to induce antigen-specific T cells. The nonadherent cells were then stimulated with irradiated (10 Gy) adherent cells that were pulsed with HER-2/*neu* peptides at 50 μg/ml for 90 min or with PBS alone.[13] Cultures were incubated in a humidified incubator at 37°C in 5% CO_2, and cytokine production was measured.

Proliferation assay

For cell proliferation assays, 20×10^5 lymphocytes isolated from lymph nodes of women with breast cancer were cultured in triplicate in 96-well plates in 200 μl as follows: (1) with each peptide at 50 μg/ml, (2) with tetanus toxoid at 5 μg/ml, (3) with phytohemagglutenin (PHA) (GIBCO Life Technologies, Grand Island, NY) for 96 h, or (4) without peptide.[18,19] During the final 16 h of culture, 1 μCi tritium-labeled thymidine ^{3}H-Tdr was added to measure proliferation. The cells were then harvested, and the radioactivity was measured in a Beckman

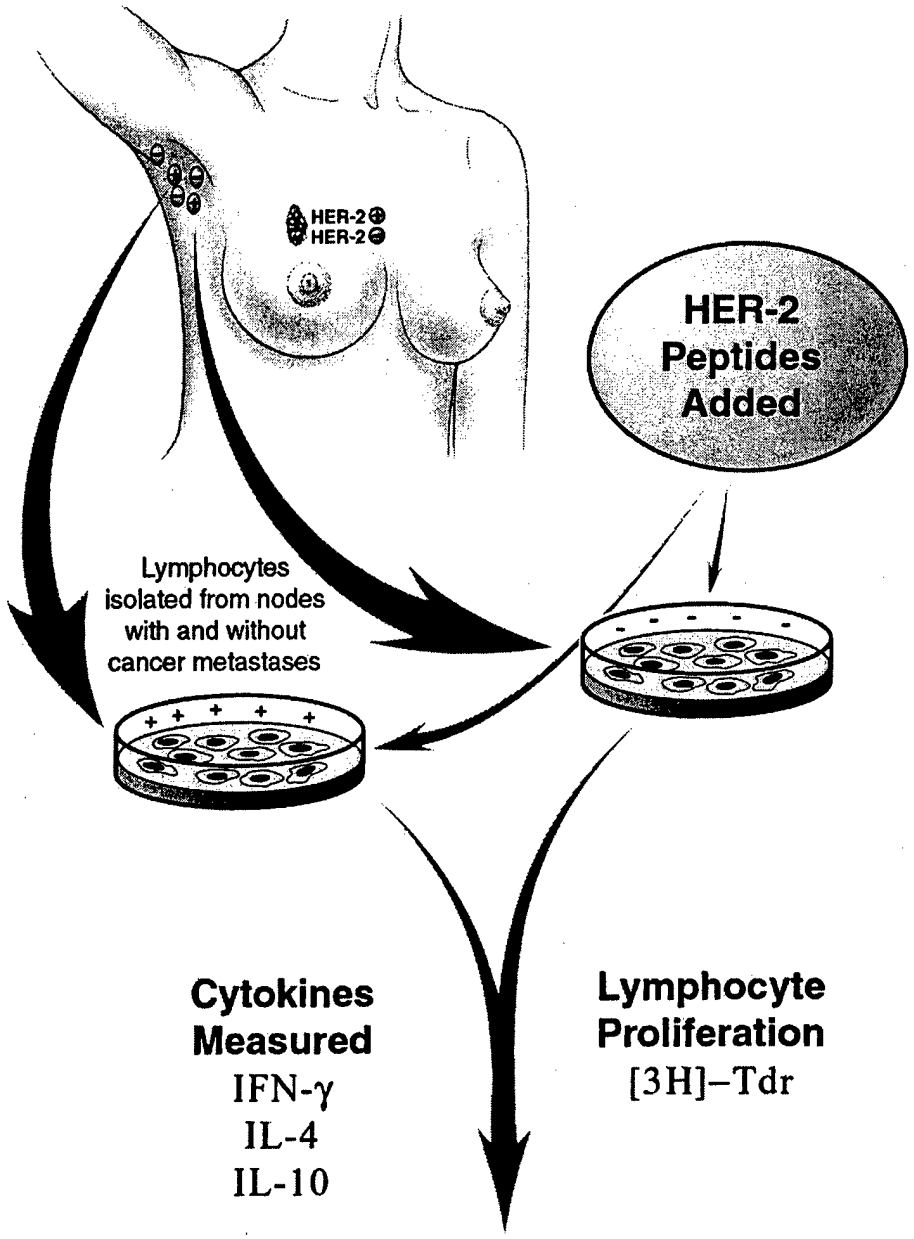


FIG. 1. Experimental design.

LS3501 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA).[20]

Cytokine production

For cytokine production assays, lymphocytes isolated from axillary lymph nodes were cultured either without or with peptides. Supernatants were collected after 48 h and stored frozen at −20°C until assayed for cytokine levels. The cytokines interferon-γ (IFN-δ), IL-4, and IL-10 were measured by double-sandwich, enzyme-linked immunosorbent assay (ELISA) using the corresponding kits (Biosource International, Camarillo, CA).

Statistical analysis

Values obtained for ^{3}H-Tdr incorporation by the lymph node lymphocytes incubated with PBS, PHA, or HER-2 peptides or without peptide were compared using the Student's *t*-test, and differences between groups were considered significant when $p < 0.05$.

RESULTS

Seven women with stage II or III breast cancer were enrolled in the study (Table 1). All patients had invasive ductal breast

TABLE 1. PATIENT AND TUMOR CHARACTERISTICS

Patient number[a]	*Disease stage*	*Tumor histology*	*No. of lymph nodes with metastases*	*HER-2/*neu
1	II	Invasive ductal carcinoma	1	Positive
2	III	Invasive ductal carcinoma	1	Negative
3	III	Invasive ductal carcinoma	2	Positive
4	II	Invasive ductal carcinoma	10	Positive
5	II	Invasive ductal carcinoma	1	Positive
6	II	Invasive ductal carcinoma	1	Negative
7	III	Invasive ductal carcinoma	0	Negative

[a]Patients 3, 4, 6, and 7 received neoadjuvant chemotherapy prior to surgery.

carcinoma. Four patients received doxorubicin-based neoadjuvant chemotherapy prior to surgery. Five patients were found to have axillary lymph node metastases at the time of axillary lymph node dissection. Four patients had HER-2/*neu*-overexpressing tumors.

*Lymphocyte proliferation following stimulation with HER-2/*neu *peptides*

Local axillary lymph node immune responses were measured by the ability of lymphocytes isolated from women with breast cancer to proliferate in response to stimulation with various HER-2/*neu* peptides (Table 2 and Fig. 2). Lymphocytes isolated from lymph nodes of women with HER-2/*neu*-overexpressing breast cancer can respond with proliferation following stimulation with the HER-2/*neu* peptide G89. Proliferative responses were seen in lymphocytes isolated from lymph nodes with and without metastases in a patient with a HER-2/*neu*-overexpressing tumor (Table 2, patient 1). Furthermore, proliferative responses were seen in lymphocytes isolated from lymph nodes with and without metastases in a patient whose tumor did not overexpress HER-2/*neu* (Table 2, patient 2, and Fig. 2A), which suggests prior immunity to this oncogenic protein. In a third patient, whose tumor overexpressed HER-2/*neu*, proliferative response was seen in lymphocytes from metastasis-free nodes but not in lymphocytes from metastasis-containing nodes after stimulation with G89 (Table 2, patient 3, and Fig. 2B). These experiments illustrate the heterogeneity of the human immune response in patients with and without axillary metastases with and without HER-2/*neu*-overexpressing breast tumors.

In patient 2 (Fig. 2A), lymphocytes isolated from a lymph node without metastases demonstrated significant proliferation after stimulation with F7 and G89. Lymphocytes isolated from the same patient from a lymph node with metastases also demonstrated significant proliferation after stimulation with F7 and G89. However, this response appeared to be blunted compared with the response seen in lymphocytes from the lymph node with metastases ($p < 0.05$). In patient 3 (Fig. 2B), lymphocytes isolated from a lymph node without metastases demonstrated significant proliferation after stimulation with F7 and G89 ($p < 0.05$). Lymphocytes isolated from the same patient from a lymph node with metastases failed to show proliferation after stimulation with F7 and G89.

*Induction of cytokine production after stimulation with HER-2/*neu *peptides*

In patients 2 and 3, secretion of IFN-γ was significantly higher than secretion of IL-4 after stimulation with the HER2/*neu* peptides F7 and G89 regardless of whether the tumor overexpressed HER-2/*neu*. Furthermore, in both patients, the ratio of secreted IFN-γ/IL-4 was significantly greater in the lymph nodes without metastases than in the lymph nodes with metastases (Fig. 3). These results suggest that lymphocytes isolated from different lymph nodes from the same patient will re-

TABLE 2. LYMPH NODE LYMPHOCYTE PROLIFERATIVE RESPONSE TO HER-2/*neu* PEPTIDE G89 (777-789) IN BREAST CANCER PATIENT AXILLARY NODES

Patient number	*Tumor HER-2/*neu *status*	*Proliferative response*[a]	
		Node without metastases	*Node with metastases*
1	Positive	+	+
2	Negative	+	+
3	Positive	+	−
4	Positive	NA	+
5	Positive	−	NA
6	Negative	+	NA
7	Negative	−	NA

[a]Responses not significantly different from those in the control groups are designated −. Significant proliferative responses are designated +. NA, not available.

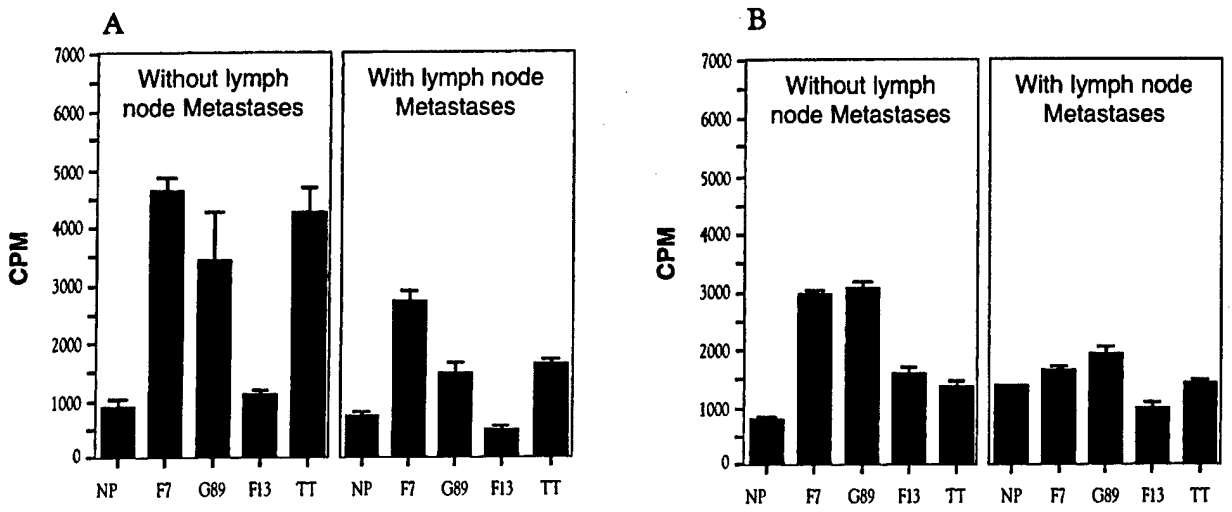


FIG. 2. Matched lymphocyte proliferative responses to HER-2/*neu* peptides (F7, G89, F13) from axillary lymph nodes with and without breast cancer metastases. (**A**) Patient 2. (**B**) Patient 3. Results expressed as cpm ± SD. NP, no peptide; TT, tetanus toxoid.

spond qualitatively differently depending on the local presence or absence of breast cancer metastases. The HER-2/*neu* peptides F7, G89, and F13 induced a predominantly Th1 pattern of cytokine production in lymphocytes isolated from lymph nodes with and without breast cancer metastases.

*IL-12-induced amplification of Th1 cytokine production after stimulation with HER-2/*neu *peptides*

In patient 6, whose tumor was not found to overexpress HER-2/*neu*, a marked proliferative response was demonstrated after G89 peptide stimulation (Fig. 4A). In this patient, IL-4 production was virtually identical after stimulation with G89 in the presence and absence of IL-12 (Fig. 4B). On the other hand, IFN-γ production was significantly increased after stimulation with G89 and IL-12 costimulation (Fig. 4C) ($p < 0.05$). In the presence of IL-12 costimulation, the ratio of IFN-γ/IL-4 secretion was markedly increased after stimulation with G89 (Fig. 4D) ($p < 0.05$). Costimulation with IL-12 produced a predominant Th1 pattern of cytokine production in lymphocytes isolated from an axillary lymph node without evidence of breast cancer metastases.

IL-12 also induced amplification of a Th1 cytokine response with HER-2/*neu* peptides in lymphocytes from patient 5, which

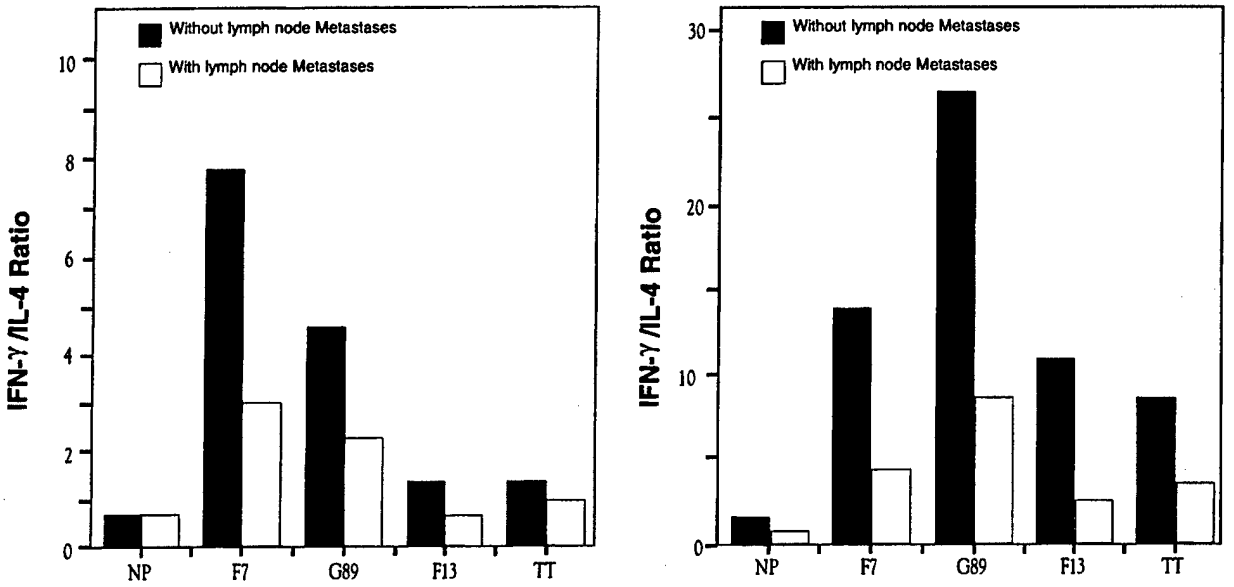


FIG. 3. IFN-γ and IL-4 secretion in lymphocytes from axillary lymph nodes with and without breast cancer metastases following stimulation with HER-2/*neu* peptides. (**A**) Patient 2. (**B**) Patient 3. NP, no peptide; TT, tetanus toxoid.

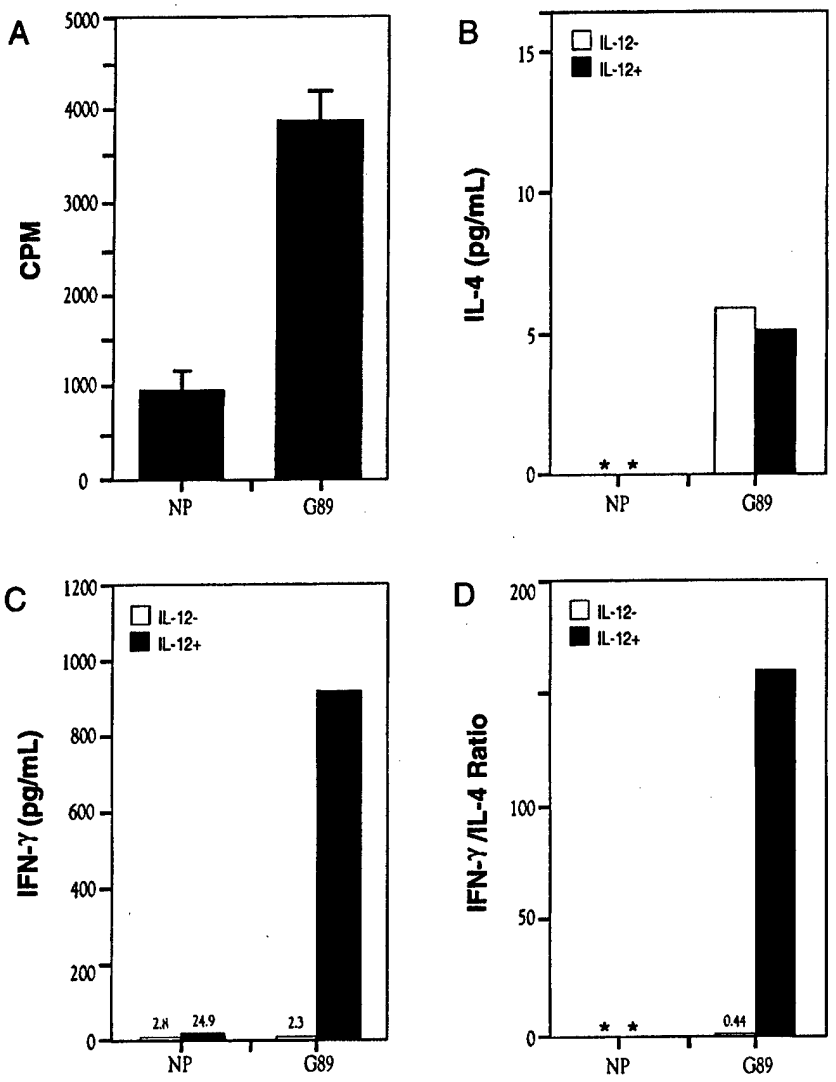


FIG. 4. IL-12-induced amplification of Th1 cytokine response with HER-2/*neu* peptide G89 stimulation in patient 6. **(A)** Proliferative response after stimulation with HER-2/*neu* peptide G89 in lymph node lymphocytes from an axillary node without metastases. **(B)** IL-4 concentration after stimulation in the presence and absence of IL-12. **(C)** IFN-γ concentration after stimulation in the presence and absence of IL-12. **(D)** Ratio of IFN-γ/IL-4 after stimulation with HER-2/*neu* peptide G89 in the presence and absence of IL-12 costimulation. NP, no peptide; **IL-4 levels below the sensitivity of detection method.

failed to demonstrate proliferation after stimulation with these peptides (Fig. 5). In the presence of IL-12, IL-4 production was significantly decreased after stimulation with the HER-2/*neu* peptides G89, G90, and E75 compared with peptide stimulation without IL-12 (Fig. 5A) ($p < 0.05$). In contrast, IL-12 induced IFN-γ secretion after stimulation with the HER-2/*neu* peptides G89 and E75 (Fig. 5B). In the absence of IL-12 costimulation (Fig. 5C), the ratio of IFN-γ/IL-4 secretion was <10 after treatment with the various HER-2/*neu* peptides, PHA, and tetanus toxoid. In the presence of IL-12 costimulation (Fig. 5D), the ratio of IFN-γ/IL-4 secretion was markedly increased after stimulation with HER-2/*neu* peptides. Costimulation with IL-12 produces a predominant Th1 pattern of cytokine production in lymphocytes isolated from this axillary lymph node without breast cancer metastases. We found that a significant Th1 cytokine response to HER-2/*neu* peptides could occur in the absence of a detectable proliferative response with these same peptides (Fig. 5). This suggests the possibility of split tolerance of lymphocytes in response to HER-2/*neu* peptide stimulation. This phenomenon may, therefore, be a characteristic immunogenic property of HER-2/*neu*-overexpressing tumors in certain patients.

*Persistence and specificity of Th1 cytokine response at priming and restimulation with HER-2/*neu *peptides*

Day 7 cultures of lymph node lymphocytes that were not initially stimulated with HER-2/*neu* peptides demonstrated a significantly higher IFN-γ/IL-10 production ratio with HER-2/*neu* peptide G89 compared with responders only, stimulators only, no peptide, and HER-2/*neu* peptide G90 (negative control) (Fig. 6A). The results of day 7 cultures of lymph node lymphocytes

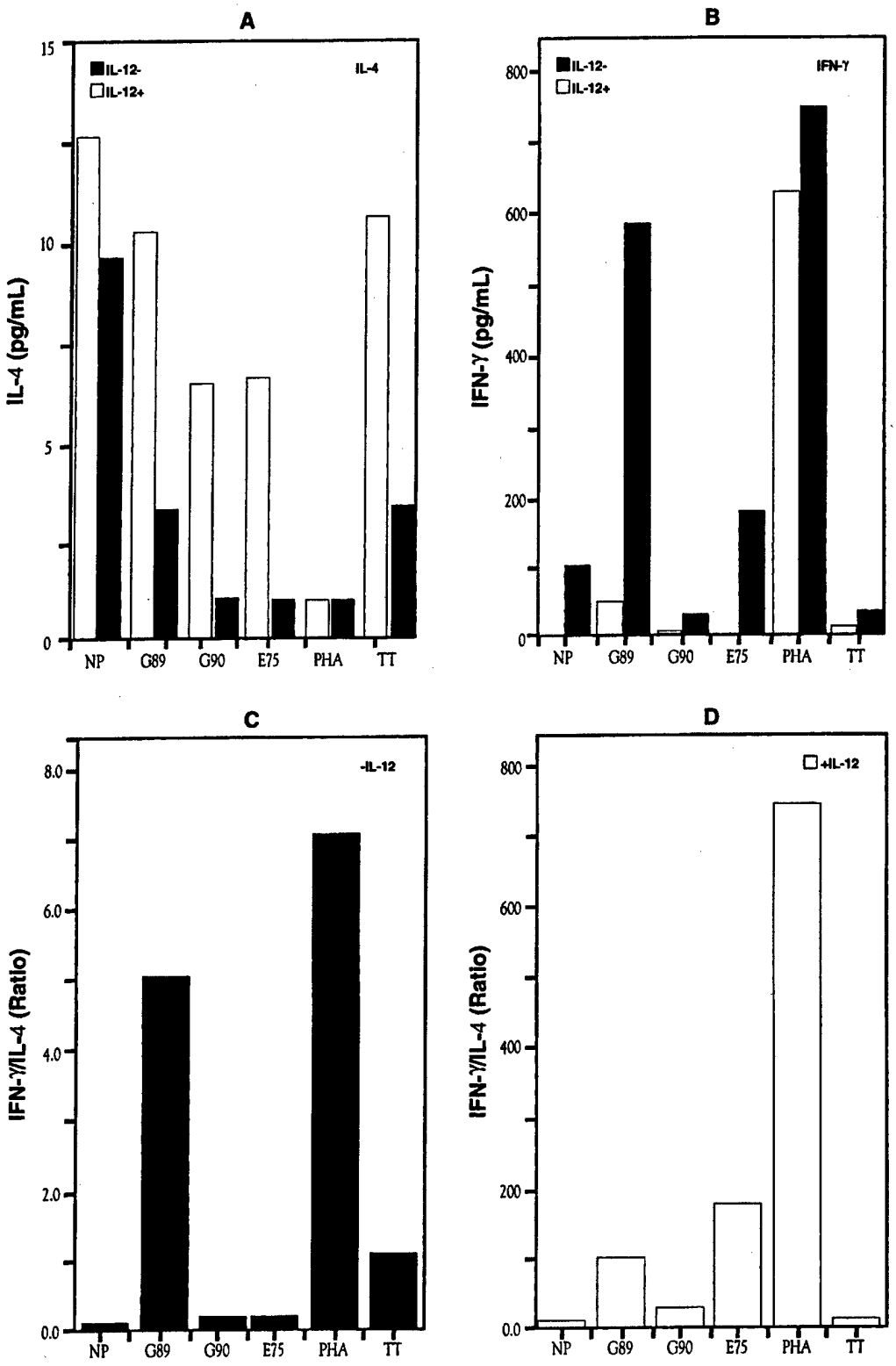


FIG. 5. IL-12 induced amplification of Th1 cytokine response with HER-2/*neu* peptides G89 and E75 in patient 5, whose lymphocytes did not demonstrate a proliferative response to these peptides. (**A**) IL-4 concentration after stimulation in the presence and absence of IL-12. (**B**) IFN-γ concentration after stimulation in the presence and absence of IL-12. (**C**) Ratio of IFN-γ/IL-4 after stimulation in the absence of IL-12 costimulation. (**D**) Ratio of IFN-γ/IL-4 after stimulation in the presence of IL-12 costimulation. NP, no peptide; TT, tetanus toxoid.

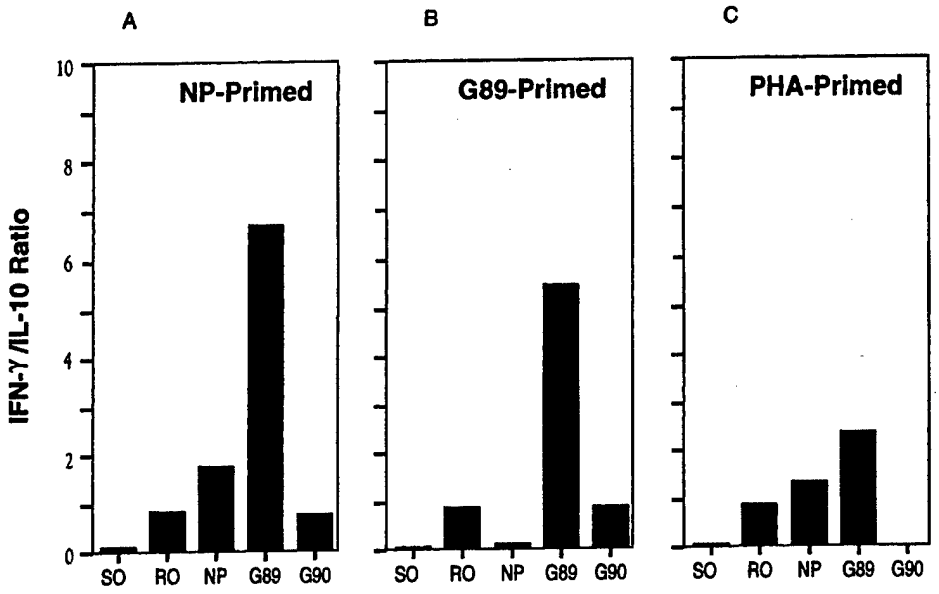


FIG. 6. Induction of Th1 cytokine response in lymphocytes isolated from an axillary lymph node with breast cancer metastases (patient 4) at priming and at restimulation with the HER-2/*neu* peptide G89. This patient had a primary breast tumor with overexpression of the HER-2/*neu* oncogene. NP, no peptide; RO, responders only (nonadherent nodal cells); SO, stimulators only (plastic adherent cells).

that were initially primed with HER-2/*neu* peptide G89 are shown in Figure 6B. Restimulation with HER-2/*neu* peptide G89 resulted in a significantly higher IFN-γ/IL-10 production ratio compared with responders only, stimulators only, no peptide, and HER-2/*neu* peptide G90 (negative control). This cytokine profile was consistent with a predominant Th1 response and was maintained after restimulation. Lymphocytes restimulated with HER-2/*neu* peptide G89 showed a significantly higher IFN-γ/IL-10 production ratio than responders only, stimulators only, and no peptide (Fig. 6C). We found that a specific and detectable HER-2/*neu* peptide Th1 response could be obtained at restimulation in previously mitogen-primed lymph node lymphocytes.

DISCUSSION

This past century has seen a great a deal of progress in the treatment of breast cancer. However, we are only at the very beginning of our understanding of the nature of the innate immunologic mechanisms that may prevent and control the development and progression of invasive carcinoma in many patients. One of the most significant recent clinically relevant developments in our understanding of breast cancer was the identification and characterization of the HER-2/*neu* oncogene product.

In this report, we have described the development of a novel *ex vivo* human model for investigating the local axillary lymph node immune response to HER-2/*neu* peptides in breast cancer patients. We believe that the information presented here not only is of theoretical interest but also may be of significant value in the appropriate design and implementation of the next wave of clinical vaccine trials in breast cancer patients.

We have demonstrated that lymphocytes isolated from the draining axillary lymph nodes of breast cancer patients can mount a significant cellular immune response to HER-2/*neu* peptides manifested by proliferation or specific cytokine elaboration. Proliferative responses to HER-2/*neu* peptides were seen in tumors with and without overexpression of the HER-2/*neu* tumor oncogenic protein. In some cases, proliferative responses to HER-2/*neu* peptides in lymphocytes from lymph nodes with metastases were absent or blunted compared with the response in lymphocytes from lymph nodes without metastases from the same patient. HER-2/*neu* peptides induced a predominantly Th1 pattern of cytokine response in axillary nodal lymphocytes isolated from breast cancer patients, and this response was preserved in women who had received neoadjuvant chemotherapy. A Th1-specific cytokine production pattern was maintained after priming and restimulation with HER-2/*neu* peptides.

On the basis of the experiments described here, it is apparent that HER-2/*neu* peptides can induce potent proinflammatory T cell responses in women with breast carcinoma. This existent specific Th1 pattern of immune response associated with *ex vivo* stimulation with HER-2/*neu* peptides suggests that *in vivo* boosting with the same HER-2/*neu* peptides can augment this immune response. It has also been shown that HER-2/*neu*-specific antibodies exist in breast cancer patients and correlate with the presence of HER-2/*neu*-positive cancers.[21] This implies that immunity to HER-2/*neu* develops as a result of exposure of patients to HER-2/*neu* protein expressed by their own cancer.

It is usually not possible to induce an immune response by

immunizing with self-proteins. The mechanism associated with this phenomenon prevents autoimmune disease, appears to be mediated by T cells, and is called tolerance.[22] However, use of peptide-based vaccines in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) appears to be very effective in generating immune responses to self-proteins in humans, including HER-2/*neu* peptides.[23] Our finding in the present study that IL-12 was able to induce amplification of the Th1 immune response after stimulation with HER-2/*neu* peptides suggests that this cytokine might play an important clinical role in the design of future HER-2/*neu* peptide-based vaccine strategies.

The ability of axillary lymph node lymphocytes isolated from breast cancer patients to proliferate in response to HER-2/*neu* peptides regardless of the HER-2/*neu* status of the primary tumor suggests prior immunity to the HER-2/*neu* protein. This immunity may be related to autoimmunity to self-protein or loss of HER-2/*neu* expression during tumor progression from *in situ* carcinoma to frankly invasive breast carcinoma. In this regard, it has been established that HER-2/*neu* protein expression is much more common in ductal carcinoma *in situ* than in invasive carcinoma.[2,5,6] Although HER-2/*neu* peptides induce a predominantly Th1 pattern of cytokine production in lymphocytes isolated from lymph nodes with and without breast cancer metastases, axillary metastases occur despite some immune response with HER-2/*neu* peptides. Thus, a proinflammatory immune response does not appear to be sufficient to induce eradication of metastatic axillary nodal deposits. Taken together, these results suggest that the mechanisms associated with the local human immune response to HER-2/*neu*-specific peptides are complex and suggest that HER-2/*neu* peptides may be of value in breast cancer vaccine development.

ACKNOWLEDGMENTS

This work was supported by grants DAMD17-94-J4313 and 17-97-17098 from Core Grant CA16662.

REFERENCES

1. SLAMON, D.J., and CLARK, G.M. (1988). Amplification of c-erB-2 and aggressive human breast tumors? Science **240,** 1795–1798.
2. SAHIN, A.A. (2000). Biologic and clinical significance of HER-2/*neu* (cerbB-2) in breast cancer. Adv. Anat. Pathol. **7,** 158–166.
3. SLAMON, D.J., CLARK, G.M., WONG, S.G., LEVIN, W.J., ULLRICH, A., and McGUIRE, W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science **235,** 177–182.
4. SLAMON, D.J., GODOLPHIN, W., JONES, L.A., HOLT, J.A., WONG, S.G., KEITH, D.E., LEVIN, W.J., STUART, S.G., UDOVE, J., ULLRICH, A., and PRESS, M.F. (1989). Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science **244,** 707–712.
5. VAN DE VIJVER, M.J., PETERSE, J.L., MOOI, W.J., WISMAN, P., LOMANS, J., DALESIO, O., and NUSSE, R. (1988). Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma *in situ* and limited prognostic value in stage II breast cancer. N. Engl. J. Med. **319,** 1239–1245.
6. ESSERMAN, L.J., LOPEZ, T., MONTES, R., BALD, L.N., FENDLY, B.M., and CAMPBELL, M.J. (1999). Vaccination with the extracellular domain of p185*neu* prevents mammary tumor development in *neu* transgenic mice. Cancer Immunol. Immunother. **47,** 337–342.
7. PRESS, M.F., CORDON-CARDO, C., and SLAMON, D.J. (1990). Expression of the HER-2/*neu* proto-oncogene in normal human adult and fetal tissues. Oncogene **5,** 953–962.
8. SLAMON, D.J., LEYLAND-JONES, B., SHAK, S., FUCHS, H., PATON, V., BAJAMONDE, A., FLEMING, T., EIERMANN, W., WOLTER, J., PEGRAM, M., BASELGA, J., and NORTON, L. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl. J. Med. **344,** 783–792.
9. IOANNIDES, C.G., IOANNIDES, M.G., and O'BRIAN, C.A. (1992). T-cell recognition of oncogene products: a new strategy for immunotherapy. Mol. Carcinog. **6,** 77–82.
10. FISK, B., BLEVINS, T.L., WHARTON, J.T., and IOANNIDES, C.G. (1995). Identification of an immunodominant peptide of HER-2/*neu* proto-oncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J. Exp. Med. **181,** 2109–2117.
11. ANDERSON, B.W., KUDELKA, A.P., HONDA, T., POLLACK, M.S., GERSHENSON, D.M., GILLOGLY, M.A., MURRAY, J.L., and IOANNIDES, C.G. (2000). Induction of determinant spreading and of Th1 responses by *in vitro* stimulation with HER-2 peptides. Cancer Immunol. Immunother. **49,** 459–468.
12. TUTTLE, T.M., ANDERSON, B.W., THOMPSON, W.E., LEE, J.E., SAHIN, A., SMITH, T.L., GRABSTEIN, K.H., WHARTON, J.T., IOANNIDES, C.G., and MURRAY, J.L. (1998). Proliferative and cytokine responses to class II HER-2/*neu*-associated peptides in breast cancer patients. Clin. Cancer Res. **4,** 2015–2024.
13. FISK, B., HUDSON, J.M., KAVANAGH, J., WHARTON, J.T., MURRAY, J.L., IOANNIDES, C.G., and KUDELKA, A.P. (1997). Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res. **17,** 45–53.
14. IOANNIDES, C.G., FISK, B., JEROME, K.R., IRIMURA, T., WHARTON, J.T., and FINN, O.J. (1993). Cytotoxic T cell from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J. Immunol. **151,** 3693–3703.
15. FALK, K., ROTZSCHKE, O., STEVANOVIC, S., JUNG, G., and RAMMENSEE, H.G. (1994). Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. Immunogenetics **39,** 230–242.
16. CHEN, L., CHEN, D., BLOCK, E., O'DONNELL, M., KUFE, D.W., and CLINTON, S.K. (1997). Eradication of murine bladder carcinoma by intratumor injection of a bicistronic adenoviral vector carrying cDNAs for the IL-12 heterodimer and its inhibition by the IL-12 p40 subunit homodimer. J. Immunol. **159,** 351–359.
17. SIDERS, W.M., WRIGHT, P.W., HIXON, J.A., ALVORD, W.G., BACK, T.C., WILTROUT, R.H., and FENTON, R.G. (1998). T cell- and NK cell-independent inhibition of hepatic metastases by systemic administration of an IL-12-expressing recombinant adenovirus. J. Immunol. **160,** 5465–5474.
18. DISIS, M.L., CALENOFF, E., McLAUGHLIN, G., MURPHY, A.E., CHEN, W., GRONER, B., JESCHKE, M., LYDON, N., McGLYNN, E., LIVINGSTON, R.B., MOE, R., and CHEEVER, M.A. (1994). Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res. **54,** 16–20.
19. ELSON, C.J., BARKER, R.N., THOMPSON, S.J., and WILLIAMS, N.A. (1995). Immunologically ignorant autoreactive T cells, epitope spreading and repertoire limitation. Immunol. Today **16,** 71–76.
20. IOANNIDES, C.G., PLATSOUCAS, C.D., and FREEDMAN, R.S.

(1990). Immunological effects of tumor vaccines: II. T cell responses directed against cellular antigens in the viral oncolystates. In Vivo **4,** 17–24.

21. DISIS, M.L., PUPA, S.M., GRALOW, J.R., DITTADI, R., MENARD, S., and CHEEVER, M.A. (1997). High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. J. Clin. Oncol. **15,** 3363–3367.
22. KAMRADT, T., and MITCHISON, N.A. (2001). Tolerance and autoimmunity. N. Engl. J. Med. **344,** 655–664.
23. DISIS, M.L., GRABSTEIN, K.H., SLEATH, P.R., and CHEEVER, M.A. (1999). Generation of immunity to the HER-2/*neu* oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin. Cancer Res. **5,** 1289–1297.

Address reprint requests to:
Dr. Henry M. Kuerer
Department of Surgical Oncology
The University of Texas M.D. Anderson Cancer Center
1515 Holcombe Boulevard
Houston, TX 77030

Tel: (713) 745-5043
Fax: (713) 792-4689
E-mail: hkuerer@mdanderson.org

Received 14 January 2002/Accepted 28 January 2002

Cancer Immunol Immunother (2000) 49:459–468

ORIGINAL ARTICLE

**Brett W. Anderson · Andrezj P. Kudelka
Toshie Honda · Marilyn S. Pollack
David M. Gershenson · Michael A. Gillogly
James L. Murray · Constantin G. Ioannides**

Induction of determinant spreading and of Th1 responses by in vitro stimulation with HER-2 peptides

Received: 10 April 2000 / Accepted: 12 July 2000

Abstract Immunization with tumor antigens induces cellular and humoral immune responses. These responses by T cells are specific for defined epitopes (determinants) in the molecule of the immunizing tumor antigen. Extension of such responses to self-antigens requires induction of autoimmunity to the tumor. As with systems of autoimmune disease, expression of T cell autoimmunity is charaterized by diversification of responses from the inducer determinant to other responder (cryptic) determinants. Since similar strategies may be useful for therapy of human cancers, we investigated whether the induction of response to a HER-2 peptide F7 (776–789) induces enhanced reactivity of other HER-2 peptides. We found that stimulation with F7 can expand a response to another epitope F13 (884–899) in both an ovarian cancer patient with progressive disease and a healthy donor who shared HLA-DR11. This response was characterized mainly by increased interferon γ secretion, and proliferation, but was not observed with another donor who shared HLA-DR14 and HLA-DQ5 with the patient. Since repeated vaccination with the same epitope may lead to a decline of primary cell reactivity caused by apoptosis spreading the response to other epitopes, the tumor antigen may provide an approach for maintaining an inflammatory Th1 response during cancer vaccination.

Key words HER-2 · Proliferation · Th1 · Cryptic determinants

B. W. Anderson · T. Honda · D. M. Gershenson
C. G. Ioannides (✉)
Department of Gynecologic Oncology,
The University of Texas M. D. Anderson Cancer Center,
1515 Holcombe Boulevard, Box 67,
Houston, Texas 77030, USA

M. A. Gillogly · J. L. Murray
Department of Bioimmunotherapy,
The University of Texas M. D. Anderson Cancer Center,
Houston, Texas, USA

A. P. Kudelka
Department of Gynecologic Medical Oncology,
The University of Texas M. D. Anderson Cancer Center,
Houston, Texas, USA

M. S. Pollack
Histocompatibility Laboratory, Department of Surgery,
University of Texas Health Science Center,
San Antonio, Texas 78284, USA

C. G. Ioannides
Department of Immunology,
The University of Texas M. D. Anderson Cancer Center,
Houston, Texas, USA

Introduction

Studies during recent years have identified tumor antigens that are targets of tumor-reactive cytotoxic T lymphocytes (CTL; reviewed by Boon and van der Bruggen [2]). These antigens are self-proteins that are capable of inducing a cellular response (mediated by $CD8^+$ or $CD4^+$ cells) and/or a humoral response. Recent experimental findings support the concept that CTL and $CD4^+$ cells recognizing tumor antigens can mediate tumor regression and justify the design and development of epitope-directed cancer vaccines [14, 17]. For all tumor antigens the major issue that needs to be resolved is how to generate and optimize an immune response to the tumor. Although both CTL and helper T cells have been identified that recognize peptide epitopes from proteins such as HER-2, gp100, MART-1, p53, etc. [21] and could be easily expanded in vitro [12], the disease progressed in patients, suggesting that either the detected response is too weak to control cancer spread, or the response to tumor is silenced.

Repeated stimulation with the same CTL epitope enhances antitumor CTL expansion with slow kinetics [1, 9]. Since the tumor environment is either tolerogenic or suppressive, CTL induction and expansion depend in many instances on exogenous cytokine help to promote CTL survival and maintain an inflammatory environment. Because of the short half-life of cytokines and side-effects, longer-lasting help can be also provided by

$CD4^+$ cells when they are activated by the appropriate antigen. However, repeated vaccinations with the same $CD4^+$ helper epitope are accompanied by a decline of primary T cell reactivity over time, suggesting that reactive T cells may be eliminated by apoptosis [19]. Thus, an alternative approach to repeated exogenous cytokine administration and repeated $CD4^+$ epitope application is to amplify the Th1 response by spreading [7, 8] it to other epitopes that are endowed with Th1-cytokine-secreting ability. Intramolecular epitope spreading under these circumstances should be more beneficial for an antitumor response than intermolecular spreading because it activates responders to the tumor antigen of interest rather than to irrelevant antigens, which may not be present at that time. This approach requires first the identification of both the inducer and the amplified epitope. To address these questions we reasoned that, if the responses of a cancer patient to a number of HER-2 epitopes are detectable at primary stimulation during the disease-free period, this will indicate an in vivo priming event by an epitope from HER-2 that is presented by antigen-presenting cells (APC). The decline in the responses to some epitopes during disease progression may point to epitopes that are no longer being presented, as well as to a growing tolerance by the existent responders. Analysis of the ability of these epitopes to induce diversification of responses to other epitopes, with concomitant enhancement of type 1 cytokine secretion, may define a stimulation sequence for inducing an inflammatory autoreactive response. We found that priming peripheral blood mononuclear cells (PBMC) from an ovarian cancer patient with the HER-2 peptide F7(776–789) induced diversification of this response to the HER-2 epitope F13(884–899). The response to F7 in this patient was gradually lost during the stable disease period, while the response to F13, which was present during the same period, was lost when the disease progressed. Analysis of specificity and restriction of this response in MHC-II-matched donors indicated that it was associated with HLA-DR11. These results support the hypothesis that intramolecular determinant spreading can be induced by HER-2 peptides and can lead to induction of autoimmunity to cancer antigens. The pattern of spreading identified can provide a basis for epitope selection for cancer vaccine development.

Materials and methods

PBMC

Peripheral blood was collected from two healthy donors designated as donor 1 (HLA-A2, 23, B7.48, DR7, 11, DQ2.6) and donor 2 (HLA-A11, 68, B51, 67, DR13, 14, DQ5, 6), and from an ovarian cancer patient, designated as patient 1 (HLA-A24, 28, B35, W6, 70, CW3, 4, DR11, 14, DQ5). The HLA typing for the healthy donors was performed in the blood bank of the M.D. Anderson Cancer Center, while the typing of the patient was performed by molecular methods. PBMC were separated by Ficoll-Hypaque and used for stimulation immediately after separation.

Antigens

The eight HER-2 peptides, constructed by the Synthetic Antigen Laboratory of the M.D. Anderson Cancer Center, derived from the amino acid sequence of the human HER-2. These peptides have been previously selected on the basis of the computer program ANT.FIND.M., which was used for the prediction of candidate T cell epitopes on HER-2 protein [6]. The sequences of peptides used in this study are as follows: D122 (396–406): QLQVFETLEET, F12 (449–465) GISWLGLRSRELGSGL; G88 (450–463); ISWLGLRSRELGS; F7 (776–789) GSYVSRLLGICL; G89 (777–790); SPYVSRLLGICL; F13 (884–899) VPIKWMALESILRRRF; G90 (886–898); IKWMALESILRRR and F14 (474–487): TVPWDQ LFR NPHQA. The residues that are potential HLA-DR11 anchors are in bold type. Because of the ability of peptides to bind on alternative registers and because of mutations in the MHC-class-II-binding pocket, motifs that predict specific binding to MHC class II are not yet well defined. Most of these HER-2 peptides contain a minimum of two of the three major anchors reported for the major HLA-DR types (i.e. HLA-DR4, -DR1, -DR11). A larger panel of HER-2 peptides was previously tested for the ability to induce proliferation of PBMC from randomly selected healthy donors and ovarian cancer patients [4]. Results of our previous study indicated that F7 and F13 induced proliferative responses in PBMC of healthy donors (57% and 62% respectively) and cancer patients (24% and 21% respectively) with higher frequency than did the other HER-2 peptides tested. In contrast, F12 was less frequently recognized (21% in healthy donors and 4% in cancer patients) [4]. F7 and mainly its analog G89(777–790) induced proliferation of PBMC from a large number of $HLA\text{-}DR4^+$ breast cancer patients [18]. The HER-2 intracellular and extracellular domains were gifts from Dr. Kenneth Grabstein, Corixa Corporation, and were prepared as described [20].

T cell proliferation assays

For characterization of T cell responses to HER-2 peptides, PBMC were plated into 24-well plates at a final concentration of 2×10^6/ml in complete RPMI medium supplemented with 5% human AB serum [4, 20]. Each peptide was added at a final concentration of 20 μg/ml. Equal volumes of cells were plated in quadruplicate in a 96-well plate 5 and 6 days later, incubated with 1 μCi tritiated thymidine ($[^3H]dT$) (Amersham), and counted as previously described [4, 20]. Results are expressed as the stimulation index (SI) representing the ratio between the mean radioactivity of the cultures stimulated with peptide, and the mean radioactivity of the cultures that had not been stimulated with peptide (NP). For in vitro expansion of T cell cultures, 6 days after the primary stimulation, interleukin-2 (IL-2; Cetus) was added to each culture at a final concentration of 20 Cetus U/ml for 4–5 additional days. Afterwards, over the next 5 days, IL-2 was gradually removed from these cultures. For the last 48 h before restimulation, the lymphocytes were rested by being cultured in the absence of exogenous IL-2. Restimulation experiments were performed in same way as primary stimulation with the difference that APC were X-ray irradiated (100 Gy) autologous PBMC.

Cytokine assays

The ability of PBMC to release cytokines in response to antigen stimulation was determined by culturing PBMC either as unstimulated cells (NP) or stimulated with the corresponding peptides. Supernatants were collected after 48 h and stored frozen at −20 °C until assayed for cytokine levels. Interferon γ (IFNγ), IL-4 and IL-10 were measured by double sandwich enzyme-linked immunosorbent assay (ELISA) using the corresponding kits provided by Biosource International (Camariyo, Calif.). The assays were calibrated with human recombinant IFNγ, IL-4 and IL-10 to detect each cytokine in the range of 10–10,000 pg/ml.

Results

Stimulation of PBMC from an ovarian cancer patient with HER-2 peptide F7 induced the intramolecular spreading of response to HER-2 peptide F13

The responses of patient 1 to HER-2 peptides were tested five times over a period of 21 months. Initially, when the disease was stable, the patient's PBMC responded to F7, were borderline in their response to F13 and F14, but did not respond to HER-2 peptides D122 and F12. Thus, the responses of this patient to F7, F13, and F14 were considered specific. During the following 16 months of stable disease the responses to F7 and F14 gradually declined, but the patient maintained a borderline response to F13. The proliferative response to F13 declined when the disease progressed (Fig. 1). This could be interpreted as the patient becoming selectively tolerant or as the deletion by apoptosis of cells responding to F7, F14, and F13. Alternatively, it was possible that F7 and F14 were no longer presented by APC, while F13 continued to be presented during the period when the disease was stable.

In a previous report [4] we showed that, during the stable-disease period, F13-primed cultures (a) responded to F13 with better proliferation than did F7-primed cultures to F7, and (b) showed a weak proliferative response to F7. The ratio IFNγ/IL-10 secreted from F13-primed cultures in response to polyclonal activators OKT3+TPA was higher than that from F7-primed cultures. This suggested that either these epitopes were cross-reactive, or priming with F13 enhanced the ability of F7-reactive cells to respond to F7 [4]. This also suggested that the in vivo and in vitro priming response to F7 was insufficient, in either quantitative or qualitative terms, to mediate polarization of the environment for a Th1 response. F7-reactive cells secreted either low levels of IFNγ and high levels of IL-10, or higher levels of IFNγ than IL-10 but, because their numbers were smaller than those of the responders to F13, the amount of IFNγ was not sufficient to inhibit the high background of Th2 cytokines [4].

The fact that, at the time of disease progression, responses to both F7 and F13 were no longer detected raised the possibility of addressing the ability of F7 and F13 to reverse their state of tolerance (nonresponsive) to each other, and to establish whether they could serve as inducer and amplifying epitope respectively.

PBMC collected at 21 months and stimulated with F7, F13 or F12, and control NP, showed insignificant proliferative responses. These peptides induced low levels of IFNγ at priming [NP = 37, F7 = 72, F12 (negative control) = 42, and F13 = 68 pg/ml], but the IL-10 levels were high in all cultures (above 250 pg/ml). To address whether responses to F7 and F13 were enhanced at reactivation, the cultures primed with F7 and F13 were restimulated with F7 and F13 using cultures with no peptide (NP) as control. The results in Fig. 2A show that

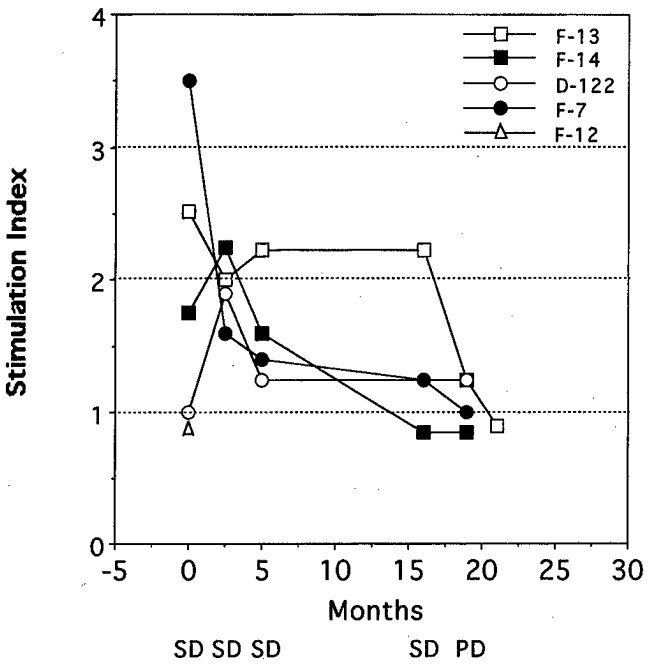


Fig. 1 Plot of proliferative responses over time for peripheral blood mononuclear cells (PBMC) from patient 1, stimulated with each of the HER-2 peptides. Stimulation indexes (SI) were calculated by comparing the proliferation ([^{3}H]dT uptake) in each independently performed experiment as described in Materials and methods. *SD* stable disease, *PD* progressive disease. The average radioactivities (cpm) for the experiments performed on months 0 and 19 were as follows. Month 0: no peptide stimulation (NP) = 595 ± 59, F7 = 2,050 ± 101, F13 = 1,498 ± 71, D122 = 560 ± 64, F14 = 1,004 ± 79; F7-, F13-, and F14-induced proliferations were all significantly higher than that of the control (NP) (595 ± 59 cpm). Month 6: NP = 744 ± 43, F7 = 943 ± 83, F13 = 1,664 ± 119, F14 = 623 ± 58. F13-induced proliferation was significantly higher than that induced by NP and F7 ($P < 0.05$). The values for the experiments performed in months 5 and 19 were in the same range as those for months 0 and 16

F13-primed cells responded to F7 and F13, while F7-primed cells responded only to F13 with SI < 2.0, but with significantly higher ^{3}H incorporation than control (NP) cultures. The small increase in proliferative response in F7F7 compared with F7NP was considered not significant. This suggested that, although F7- and F13-reactive cells were present, their numbers were too small to show a significant increase in proliferation levels.

In contrast, analysis of IFNγ and IL-4 levels showed that F7-primed cultures increased their IFNγ response twofold on restimulation and responded to F7 with slightly higher levels of IFNγ than those produced in response to F13. F13-primed cultures also responded to F7 with slightly higher levels of IFNγ than resulted in response to F13. The IL-4 levels were lower in response to both F7 and F13 than with F7- and F13-primed cultures, which were stimulated with APC only (NP). The IFNγ/IL-4 ratios were similar in both cultures. The most dramatic effect of peptide restimulation was observed with IL-10 induction. The IL-10 levels in control cultures, not stimulated with peptide at either

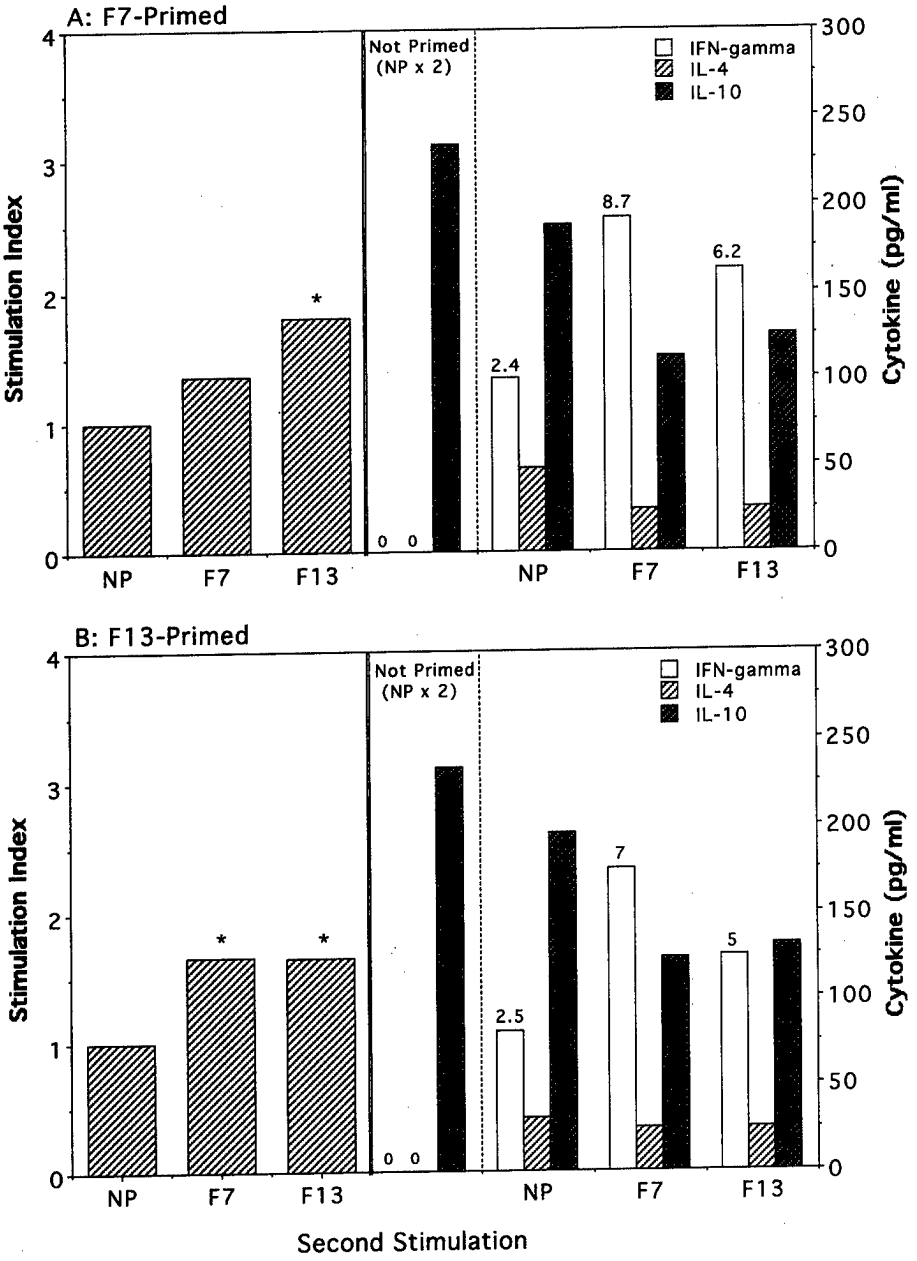


Fig. 2A, B Proliferative and cytokine responses to F7 and F13 of F7-primed and F13-primed PBMC from patient 1, collected in month 21. **A** F7-primed, **B** F13-primed cells. Samples of 4×10^4 PBMC primed with F7 or F13 were restimulated with 10^5 autologous PBMC as indicated. The concentration of exogenous pulsed peptide was 20 μg/ml. The experiment was performed in triplicate. **B** Radioactivities following restimulation of F7-primed cells with F13, and of F13-primed cells with F13 and F7 are significantly higher (*$P < 0.05$) than those for NP, but stimulation indexes are below 2.0; i.e. F7NP = 1,631 ± 96 cpm, F7F7 = 2,194 ± 187 cpm, F7F13 = 2,936 ± 140 cpm, and F13NP = 1,056 ± 70 cpm, F13F7 = 1,754 ± 129 cpm, F13F13 = 1,740 ± 99 cpm. Numbers above the open columns indicate interferon γ (*IFNγ*)/interleukin-4 (*IL-4*) ratios

priming or restimulation (NPNP), were high (234 pg/ml). Priming with F7 and F13 slightly decreased IL-10 levels to 190–200 pg/ml. Restimulation with F7 and F13 decreased IL-10 levels even further by 30%–40% (Fig. 2A, B). These results suggested that F7-primed and F13-primed cells from this patient enhanced their response at restimulation with the same peptides, since they modulated their cytokine response, but the numbers of specific responders were too low to detect significant changes in proliferation. The IFNγ/IL-10 ratios indicated that, at restimulation, F7 induced a stronger Th-1 cytokine response than F13.

Since stimulation with F7 appeared to enhance the IFNγ response to F13, we investigated whether these peptides induced spreading of response to each other. To address whether there is a reciprocal spread of proliferative responses to F7 and F13, each of the F7-primed and F7-restimulated cultures, designated F7F7) and of the F13-primed and F13-restimulated cultures (designated F13F13) was restimulated with F7 or F13. F7F7-stimulated cells showed significant proliferation in response to both F7 and F13 (Fig. 3A). This expansion of the proliferative response was accompanied by a two- to threefold increase in the IFNγ levels (Fig. 3B). In contrast, F13F13-stimulated cells showed a weaker increase in proliferative responses to either F7 or F13.

Proliferative responses with SI > 2.0 were not observed in F13F13 cells stimulated by F7 and F13 but the levels of radioactivity were still significantly higher than those of F13F13 NP cultures (Fig. 3C).

The IFNγ response paralleled the proliferative responses. The IFNγ response to F7 of both F7F7 and

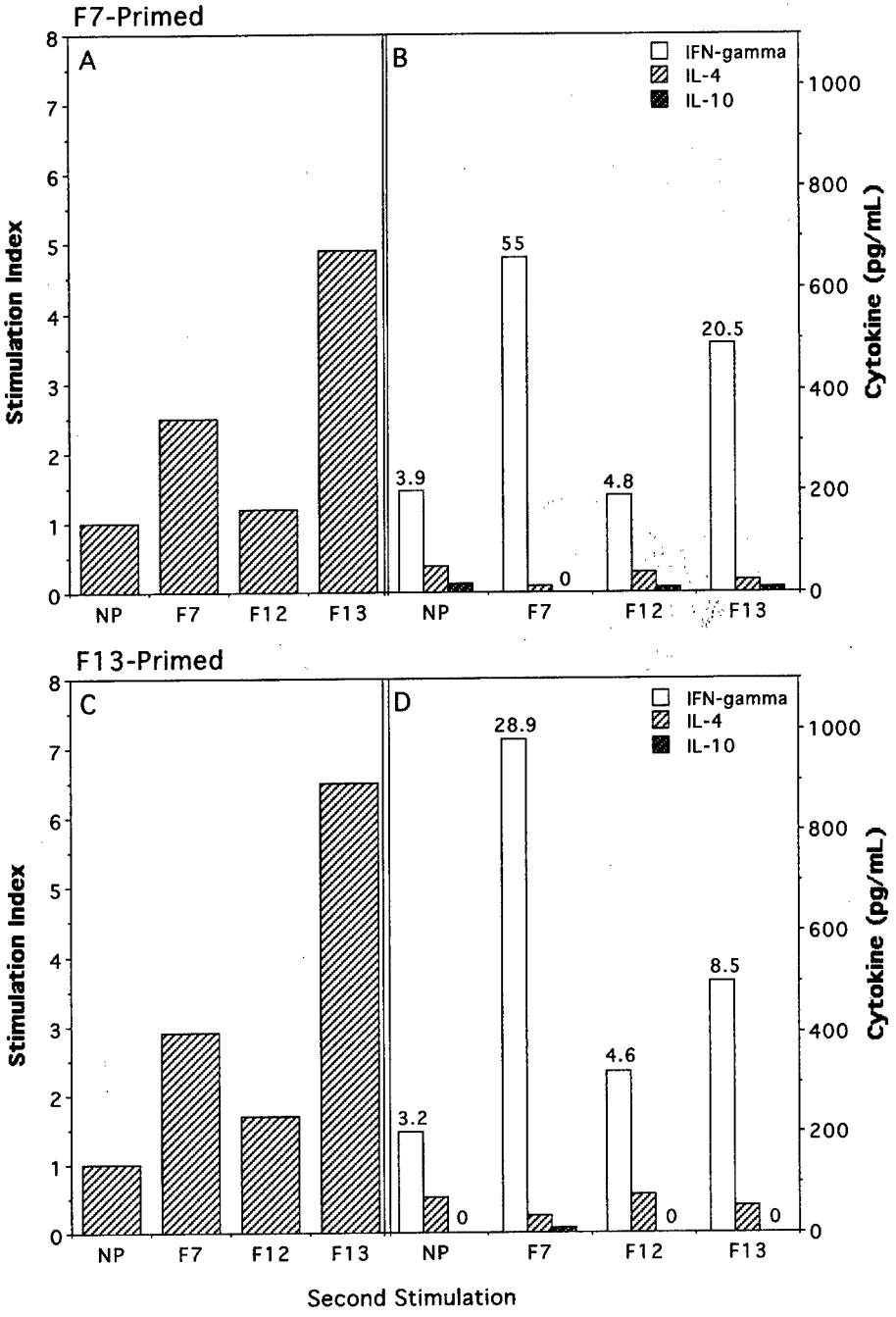


Fig. 3A–D Proliferative (**A, C**) and cytokine (**B, D**) responses of PBMC from patient 1 on third stimulation with F7 and F13. F7F7 and F13F13 indicate that responders were stimulated twice with F7 and F13. Radioactivities and SI for F7- and F13-stimulated, F13F13 cultures were significantly higher than those for NP-stimulated F13F13 cultures: F13F13 NP = 1,427 ± 59 cpm, F13F13F7 = 2,995 ± 112 cpm (SI = 2.1), F13F13F13 = 2,553 ± 61 cpm (SI = 1.80) (*$P < 0.05$). Experimental conditions as described in Fig. 2

F13F13 cells was higher than the response to F13. In addition, the IFNγ/IL-4 ratio in F7F7F13-stimulated cultures (Fig. 3B) was almost double the IFNγ/IL-4 ratio in F13F13F13-stimulated cultures (Fig. 3D). These results suggested that F7 had a stronger Th1-inducing effect than F13 in this system, and indicated that priming and restimulation with F7 (F7F7) expanded a response to F13, but priming with F13 (F13F13) was less effective in expanding the response to F7.

The requirement for F7 for inducing spreading of the response was confirmed by the fact that, in F13-primed cultures restimulated with F7, i.e. (F13F7), the SI values at the third stimulation were significantly higher than 2.0 in response to F7 (F13F7F7 = 4.45), unlike those of the control (F13F7NP = 0.85), but were not higher in response to F13 (F13F7F13 = 1.42).

Spreading of Th1 responses by F7 and F13 in healthy donors

The results from stimulation with F7 and F13 in this patient indicated that F7 induced a Th1 response and increased the ability of F13-reactive cells to respond to F13. Since this patient expressed HLA-DR11 and HLA-DR14, this raised the question of whether the response

was associated with one MHC-II product or both. HLA-DR11 (DRB1* 1101) is a split of DR5, while HLA-DR14 (DRB1* 1401) is a split of HLA-DR6. Thus, these class II antigens belong to distinct genetic and serological groups and anchors for both major HLA-DR types are present in both peptides. Since additional amounts of blood could not be obtained from this patient, because the disease progressed, the specificity analysis was performed using T cells from two partially HLA-DR-matched healthy donors.

Donor 1 shared only DR11 with patient 1 while donor 2 shared DR14 and DQ5 but not DR11 with patient 1. Therefore, if responses were found in donor 1 but not in donor 2, this would suggest that these peptides induce determinant spreading in association with DR7, 11 and DQ6. Since DR11 is expressed by the ovarian cancer patient this would confirm DR11, without excluding DR7 and DQ2. Alternatively, if responses were found in donor 2, this would point to HLA-DR14 being the presenting molecule without excluding others.

This analysis also aimed to address whether (a) T cells from healthy donors respond with a similar or a different pattern of cytokines to F7 and F13, and (b) whether F7 can induce determinant spreading in healthy donors in vitro or spreading requires prior in vivo priming with the tumor antigen during disease.

To address these questions, PBMC from donor A ($DR11^+$) were used as control (NP) cells or primed with F7, F12 and F13. Positive responses of PBMC to priming with these peptides were borderline only for F13 (NP = 816 ± 29 cpm; F7 = 779 ± 54 cpm, F12 = 893 ± 61 cpm, F13 = 1327 ± 97 cpm, F14 = 1007 ± 59 cpm. The SI indexes ranged between 0.97 (F7) and 1.63 (F13). Only the radioactivities of F13- and F14-primed cells were significantly higher than those of control NP but the overall values were low. IFNγ was detected only in response to F13 and its levels were 70 pg/ml (data not shown). Each peptide-primed culture was restimulated with NP, F7, F12 being used as a control. The results in Fig. 4A, C show that F7-primed cells responded to both F7 and F13, but with higher proliferation in response to F13 (SI > 4.5) than to F7 (SI > 2.5). These responses were mediated by $CD4^+$ cells on the basis of inhibition by anti-CD4 and anti-HLA-DR mAb (L243), but not by anti-CD8 (Table 1). In parallel, F13-primed cells also responded to F7 and F13 but with higher proliferation in response to F13 (Fig. 4C). Although the proliferative responses of both F7- and F13-primed cells to restimulation with F13 were higher than to the restimulation with F7, their IFNγ response to F7 was higher than the response to F13. These results confirmed that F7 can induce determinant spreading, as detected in patient 1. In this healthy donor, F13 could also induce determinant spreading. The specificity of this response for F7 and F13 is illustrated by the fact that proliferative responses to F12 were not observed while the increase in IFNγ was minimal. Also, priming with F12 did not diversify the response to F7 and F13 (not shown).

The patterns of IFNγ and IL-4 secretion in response to F7 and F13 were similar in both F7- and F13-primed cultures. The main distinction between these cultures was that the overall levels of IFNγ were lower in F7F7-stimulated cultures, than in F13F7-stimulated cultures. The levels of IL-4 in response to F7F7 were borderline (10–20 pg/ml), which is within the low levels of sensitivity of the method (Fig. 4B). The levels of IL-4 in response to F13F13 stimulation were higher than in response to F7F13 (Fig. 4D compared to 4B). Consequently, at similar IFNγ levels, the IFNγ/IL-4 ratios were almost two times higher in the cultures primed with F7 than in the cultures primed with F13. It should be mentioned that, because the IL-4 levels in response to F7F7 and F13F7 were low and at the lower levels of sensitivity of the ELISA method (20–50 pg/ml), the differences in the IFNγ/IL-4 ratios between these two groups may not accurately reflect higher Th1 activity in the F7F7 group than in the F13F7 group.

In contrast with patient 1, the levels of IL-10 in both F7-primed cultures and in F13-primed cultures were borderline (< 10 pg/ml) regardless of the peptide used for restimulation.

To address whether the lower SI for F7F7- and F13F7-stimulated cells were due to the lower numbers of F7-reacting precursors than of F13-reacting cells, the corresponding cultures were restimulated with F7 and F13. At the third stimulation, the SI for F7F7-stimulated cells was higher than SI for F13F13 F13-stimulated cells, but the highest increase was observed where F7F7 cultures were stimulated with F13: F13F13F13 (SI = 4.6) F7F7F7 (SI = 5.5) compared with F7F7F13 (SI = 9.8). F7F7F13 cells secreted higher levels of IFNγ than F7F7F7 cells (900 pg/ml compared with 480 pg/ml). Thus, the shift from F7 to F13 at the third stimulation allowed both high proliferation and IFNγ production by cells responding to F13. It should be mentioned that both F7-stimulated and F13-stimulated cells recognized HER-2 intracellular domain protein (data not shown).

The priming and restimulation experiments were repeated with donor 2 (who shared DR14 and DQ5 with patient 1) and expressed DR13 and DQ6. F7, F12, and F13 could induce neither proliferation at priming nor spreading of responses at restimulation in donor 2 (Fig. 5A–D). The levels of IFNγ on restimulation with F7 were also lower than those of donor 1 or patient A. Since responses were not found after restimulation, this suggested that F7 and F13 (as well as F12) are not presented in association with DR13, DR14 and DQ5 and DQ6. These results indicated that induction of inflammatory response to HER-2 peptides F7 and F13 was associated with HLA-DR11 as an antigen shared by patient and donor PBMC, although DR.7 and DQ6 cannot be excluded.

Discussion

In this study we identified a HER-2 epitope that initiated determinant spreading and an inflammatory cytokine

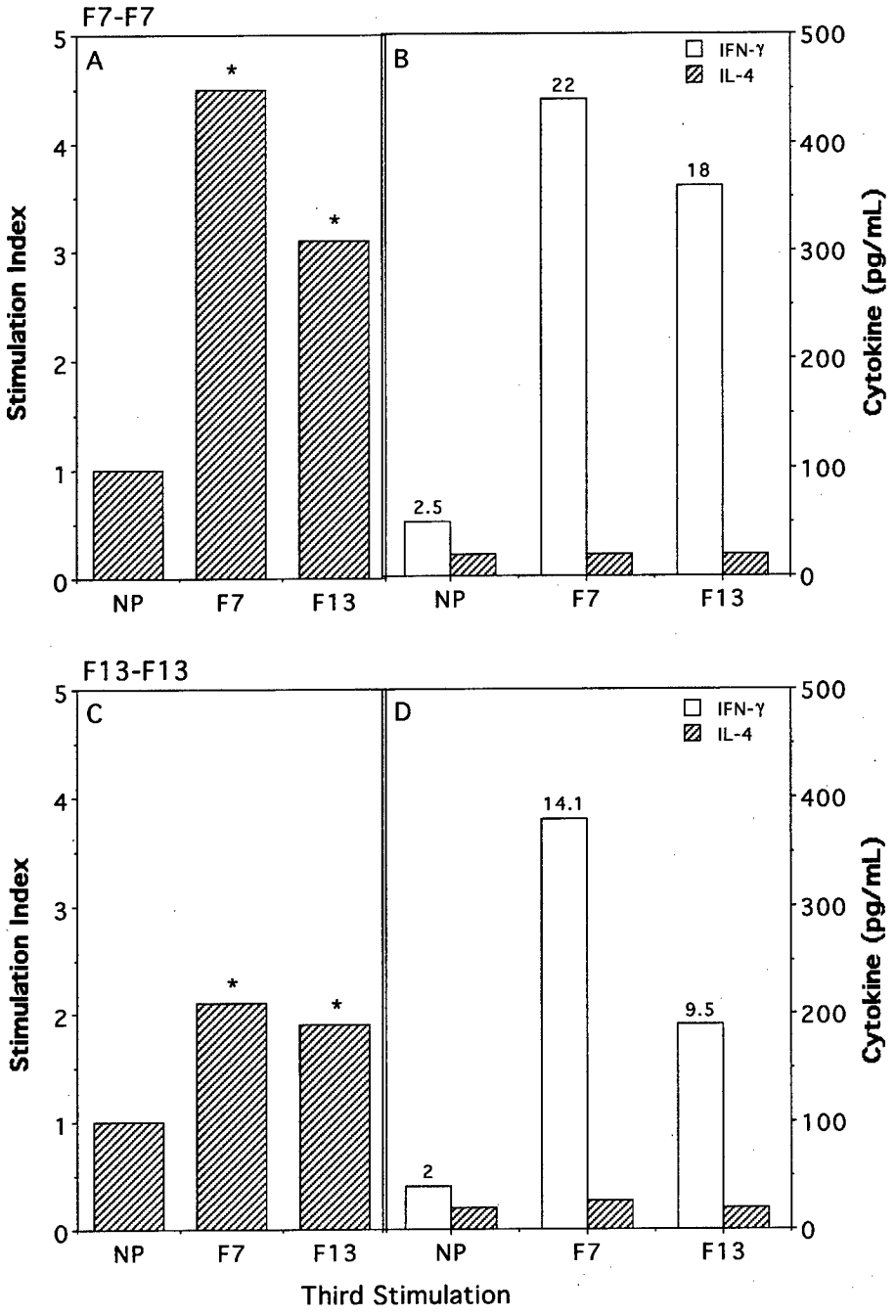


Fig. 4A–D Proliferative (**A, C**) and cytokine (**B, D**) responses of PBMC from healthy donor 1 on restimulation with F7 and F13. F12 was used as a negative control peptide. Experimental conditions are as described in Fig. 2

response in a healthy donor and an ovarian cancer patient who shared HLA-DR11. This response appeared to be restricted by HLA-DR11 since two other healthy donors who were HLA-DR14$^+$ and did not share HLA-DR11 with the patient either failed to respond to stimulation with these peptides (donor 3) or developed only a weak response. (donor 2) characterized by weak IFNγ induction and proliferation. The pattern of spreading of responses differed between the patient and the HLA-DR11-matched healthy donor. In the patient, of the two candidate Th1 peptides (F7 and F13) only F7 induced the spreading of response to F13, while, in the healthy donor, F7 and F13 each induced the spreading of the response to the other. Furthermore, in the ovarian cancer patient studied, the increase in IFNγ production, and the concomitant decrease in IL-10, preceded the detected expansion of the proliferative response. In contrast, in the healthy donor, the levels of IL-10 were low and the increase in IFNγ secretion paralleled the increase in proliferative response.

The spreading of the proliferative and cytokine response from F7 to F13 in the patient appeared to be mediated, at least in part, by the ability of F7 to modulate the cytokine profiles of the responders. The IFNγ/IL-10 ratios on restimulation of F7- and F13-primed cells F7F7, F7F13, F13F13, and F13F7 were

1.73, 1.33, 0.89, and 1.4 respectively. This suggested that priming and restimulation with F7 modulated the response towards Th1 more than priming and restimulation with F13. In contrast, in the healthy donor, not only were the levels of IFNγ high after restimulation, but the levels of IL-10 were borderline, thus facilitating the ability of F13 to condition the F7 responders or a higher IFNγ response to F7. It should be noted that, in the healthy donor, induction of inflammatory and proliferative responses to F7 and F13 required only two stimulations, while in the patient three stimulations were required to obtain the same effect. The background levels of IL-10 were severalfold higher in the cancer patient than in the healthy donor, raising the possibility that IL-10 may have down-regulated the proliferative responses to F7 and F13.

Table 1 MHC recognition by F7F7-stimulated cells. Donor 1 peripheral blood mononuclear cells (PBMC) were primed with F7 for 5 days and then expanded in a low concentration (20–40 IU/ml) of interleukin-2 (IL-2) for the following 2 weeks. Afterwards, the cells were rested for 2 days by removing IL-2. F7-primed T cells from this donor were stimulated with F7 pulsed at 20 μg/ml on 10^5 autologous irradiated (100 Gy) autologous PBMC. 20,000 responders were added to each well in the presence and absence of the indicated monoclonal antibodies. The experiment was performed in quadruplicate. [^{3}H]dT was added on day 4 for the last 16 h of incubation. Percentage inhibition was calculated by subtracting the radioactivities (cpm) for each mAb-treated culture and dividing the result by the values for the positive control (cultures stimulated with F7) minus the negative control values

mAb	F7-stimulated cells (cpm)	Peptide/SI	Inhibition (%)
–	2,019 ± 60	–/1.0	0
–	5,242 ± 57	F7/2.6	0
αCD3	2,472 ± 82	F7/1.22	87
αCD4	3,007 ± 125	F7/1.49	70
αCD8	4,990 ± 118	F7/2.47	7
αMHC-I (W6/32)	4,462 ± 97	F7/2.21	24
αMHC-II (L243)	2,836 ± 75	F7/1.40	75

The reasons for the differences between F7 and F13 in inducing proliferation and IFNγ are unclear. One possibility to be considered is that the frequency of F7-responsive cells in both the donor and the patient is significantly lower than the frequency of F13-responsive cells. In this case the increase in proliferation by F7, as measured by the thymidine incorporation, would not be detected when the numbers of responders were low, but would be detected after they had reached a significant percentage of the cells in culture. This possibility is supported by the fact that (a) F7-responding cells proliferated better at the third stimulation in both healthy donors and in the patient than did F13-responding cells, and (b) F7-primed cells responded with higher IFNγ levels than did F13-primed cells. This suggested that F7 responders recognized F7 with higher affinity than did F13 responders since they secreted higher levels of IFNγ.

The demonstration that HER-2 peptides can induce intramolecular spreading of inflammatory responses is of interest for cancer vaccine development. Introduction in vaccines of helper epitopes such as F7 can reactivate the F7-reactive cells to secrete IFNγ, and enhance the ability of F13-reactive cells to respond. Furthermore, if the response to F7 is low and/or F7 responders become tolerant, another Th1 epitope can be used as priming, followed by F7 and F13 at the next round of vaccination. Likely candidates for this function are the Th1 epitope from influenza associated with HLA-DR4 [10]

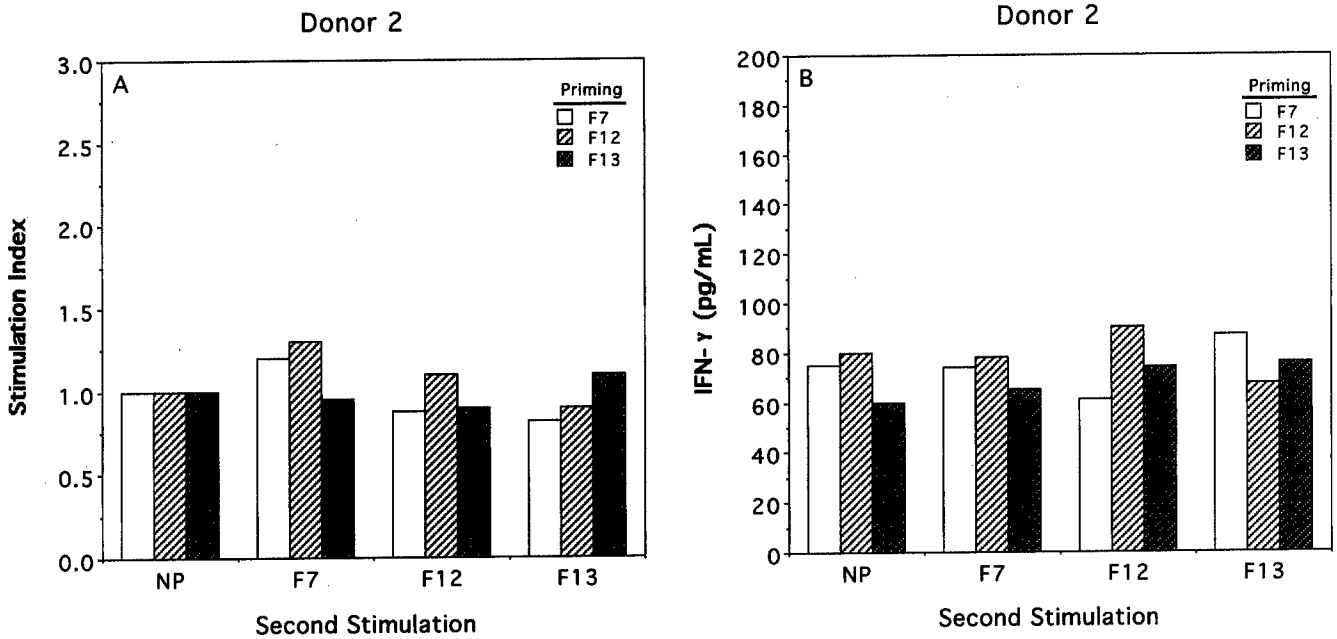


Fig. 5A, B Proliferative (**A**) and cytokine responses (**B**) of PBMC from donor 2 on restimulation with F7 and F13. SI and IFNγ levels induced by F7, F12 and F13 are not significantly different from values obtained with NP. One of the two independently performed experiments with similar results is shown. Experimental conditions as described in Fig. 2

and the helper epitope PADRE, developed by Sette and collaborators [5]. The use of defined epitopes from the same protein or of genes encoding defined epitopes should avoid induction of Th2 responses by other epitopes, that may be presented simultaneously in competition with F7 and F13. Furthermore, the successive application of Th1 epitopes in a vaccination protocol should avoid induction of apoptosis in responders by the repeated stimulation with the priming epitope. In support of this possibility Disis and collaborators recently reported that vaccination with HER-2 peptides induces both intra- and intermolecular spreading of responses in terms of proliferation.

The results also point to the fact that a peptide should be more effective in vaccination than the whole protein in inducing the spreading of a type 1 response, for several reasons. (a) Since the presentation of HER-2 epitope is still an unknown, vaccination with F7, for example, followed by F13 should focus the response to Th1 epitopes and avoid presentation of HER-2 epitopes that may induce this Th2 response. (b) Sequential peptide application may allow spread to be controlled and the response terminated when side-effects are observed. (c) The use of peptides or of the genes encoding these peptides allows lower amounts of antigen or of the viral vector to be used for vaccination. Since, on a molar basis, 100 times more HER-2 protein is needed to reach the same concentration as F7/F13, this may assist therapies using non-replicating viruses where the amount of antigen is limited.

The relationship between disease progression and decrease in response to F7 and F13 deserves further investigation. The gradual decrease in responses to F7 during the stable disease period may suggest that F7 is presented less over time, while F13 continues to be presented. The decrease in response to F13 may indicate either that F13 is no longer presented by APC to stimulate and maintain the pool of F13 responders, or that suppression of F13-responding cells by Th2 cytokines secreted by the tumor inhibits their ability to respond to F13. It is possible that F7-responding cells in this patient had a critical role in conditioning the environment or the APC for a Th1 response and were needed to sustain the ability of F13-specific cells to respond. This possibility is supported by the proposal by Steinman and collaborators that a small number of autoreactive cells in an infiltrate can control the responses of other cells to self-antigen [13]. Determinant spreading has been described as an essential component of the progressive course of autoimmune disease [3, 15, 23]. Broadening of the response to additional determinants to the primary epitope leads to more advanced autoimmune disease because of the diversified autoreactive pool of T cells. The fact that stimulation with HER-2 peptides can induce determinant spreading and Th1 responses may have important implications for development tumor immunity and for the development of cancer vaccines. While in autoimmune diseases characterized by a type 1 response, prevention of spreading is the main objective of therapy [16], in cancer, the induction of spreading of a type 1 response may be desired to induce a response against tumors. The identification of inducer and amplifying epitopes may be of interest and, for other tumor antigens recognized by $CD4^+$ cells [11, 15, 18, 22], provide a way of controlling the expansion of a therapeutic response.

Acknowledgement This work has been supported in part by the NIH-core grant CA 16 662 and the grant DAMD 17-97-I-7098 (CGI).

References

1. Anichini A, Molla A, Mortarini R, Tragni G, Bersani I, Di-Nicola M, Gianni AM, Pilotti S, Dunbar R, Cerundolo V, Parmiani G (1999) An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. J Exp Med 190: 651–6678
2. Boon T, Bruggen P van der (1996) Human tumor antigens recognized by T lymphocytes. J Exp Med 183: 725–729
3. Disis ML, Grabstein KH, Sleath PR, Cheever MA (1999) Generation of immunity to the HER-2/*neu* oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin Cancer Res 5: 1289–1297
4. Fisk B, Hudson JM, Kavanagh J, Wharton JT, Murray JL, Ioannides CG, Kudelka AP (1997) Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res 17: 45–54
5. Guercio MF del, Alexander J, Kubo RT, Arrhenius T, Maewal A, Appella E, Hoffman SL, Jones T, Valmori D, Sakaguchi K, Grey HM, Sette A (1997) Potent immunogenic short linear peptide construcs composed of B cell eiptopes and Pan DR T helper epitopes (PADRE) for antibody responses in vivo. Vaccine 15: 441–448
6. Ioannides CG, Ioannides MG, O'Brian CA (1992) T cell recognition of oncogene products. Mol Carcinog 6: 77–81
7. Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting FSP, Robinson R, Atkinson MA, Sercarz EE, Tobin AJ, Lehmann PV (1993) Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature 366–371
8. Lehmann PV, Forsthuber T, Miller A, Sercarz EE (1992) Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature 358: 156–157
9. Marincola FM, Rivoltini L, Salgaller ML, Player M, Rosenberg SA (1996) Differential anti-MART-1/MelanA CTL ability in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors. Evidence of in vivo priming by tumor cells. J Immunother 19: 266–277
10. Marshall KW, Wilson KJ, Liang J, Woods A, Zaller D, Rothbard JB (1995) Prediction of peptide affinity to HLA-DRB1*0401. J Immunol 154: 5927–5933
11. Nishimura MI, Avichezer D, Custer MC, Lee CS, Chen C, Parkhurst MR, Diamond RA, Robbins PF, Schwartzentruber DJ, Rosenberg SA (1999) MHC class I-restricted recognition of a melanoma antigen by a human $CD4^+$ tumor infiltrating lymphocyte. Cancer Res 59: 6230–6238
12. Perez-Diez A, Butterfield LH, Li L, Chakraborty NG, Economou JS, Mukherji B (1998) Generation of $CD8^+$ and $CD4^+$ T-cell response to dendritic cells genetically engineered to express the MART-1/Melan-A-gene. Cancer Res 58: 5305–5309
13. Steinman L (1996) A few autoreactive cells in an autoimmune infiltrate control a vast population of nonspecific cells: a tale of

smart bombs and the infantry. Proc Natl Acad Sci USA 93: 2253–2256
14. Surman DR, Dudley ME, Overwijk WW, Restifo NP (2000) Cutting edge: $CD4^+$ T cell control of $CD8^+$ T cell reactivity to a model tumor antigen. J Immunol 164: 562–565
15. Takahashi T, Chapman PB, Yang SY, Hara I, Vijayasandhi S, Houghton AN (1995) Reactivity of autologous $CD4^+$ T lymphocytes against human melanoma. J Immunol 154: 772–779
16. Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A, Forsthuber T, Lehmann PV, Kaufman DL (1996) Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J Exp Med 183: 1561–1567
17. Topalian SL (1999) MHC class II restricted tumor antigens and the role of $CD4^+$ T cells in cancer immunotherapy. Curr Opin Immunol 6: 741–745
18. Topalian SL, Gonzales MJ, Parkhurst M, Li YF, Southwood S, Sette A, Rosenberg SA, Robbins PF (1996) Melanoma-specific $CD4^+$ T cells recognize nonmutated HLA-DR-restricted Tyrosinase epitopes. J Exp Med 183: 1965–1971
19. Tuohy VK, Yu M, Yin L, Kawczak JA, Johnson JM, Mathisen PM, Weinstock-Guttman B, Kinkel RP (1998) The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol Rev 164: 93–100
20. Tuttle T, Anderson BW, Thompson WE, Lee JE, Sahin A, Smith TL, Grabstein KH, Wharton JT, Ioannides CG, Murray JL (1998) Proliferative and cytokine responses to class II HER-2/neu-associated peptides in breast cancer patients. Clin Cancer Res 4: 2015–2024
21. Wang RF, Rosenberg SA (1999) Human tumor antigens for cancer vaccine development. Immunol Rev 170: 85–100
22. Yoshino I, Goedegebuure PS, Peoples GE, Lee K-Y, Eberlein TJ (1994) Human tumor infiltrating $CD4^+$ T cells react to B cell lines expressing heat shock protein 70. J Immunol 153: 4149–4158
23. Yu M, Johnson JM, Tuohy VK (1996) A predictable sequential determinant spreading cascade invariably accompanies progression of experimental autoimmune encephalomyelitis: a basis for peptide-specific therapy after onset of clinical disease. J Exp Med 183: 1777–1788

Treatment with HER-2 phosphorylation agonists enhance tumor ability to stimulate epitope specific CTL *in vitro*

AGAPITO CASTILLEJA[1], NANCY E. WARD[2], RICHARD B. EPSTEIN[5], ANDRZEJ P. KUDELKA[3,4], DAVID M. GERSHENSON[1], CLAY L. EFFERSON[1], CATHERINE A. O'BRIAN[2] and CONSTANTIN G. IOANNIDES[1,2]

Departments of [1]Gynecologic Oncology, [2]Cancer Biology, [3]Gynecologic Medical Oncology and [4]Immunology, [5]Charing Cross, Westminster Medical School, University of London, London, UK

Abstract. The transmembrane (TM) receptor encoded by the HER-2 proto-oncogene (HER-2) is amplified in several types of human carcinomas and premalignant states and provides an important target for cancer therapy. While overexpression of HER-2 should lead to increased CTL epitope formation due to the attendant increase in higher protein turnover, breast tumors are poor stimulators of CTL. In this report, we show that treatment of SKBR3.A2 tumor cells with HER-2 receptor agonists (EGF and NDF) enhanced tumor ability to activate CTL from tumor associated lymphocytes (TAL) and from T cells from peripheral blood *in vitro*. The enhanced ability of tumor cells to stimulate CTL was paralleled by tyrosine phosphorylation of HER-2, and its oligo-ubiquitination compared with control untreated, or TPA-treated tumor cells. Our results demonstrate that HER-2 ligands used at concentrations which induce tyrosine phosphorylation but not downregulation of the receptor can be used to enhance the ability of tumor cells to activate CTL. This may have implications for overcoming Ag ignorance and tolerance in human cancers.

Correspondence to: Dr C.G. Ioannides, Department of Gynecologic Oncology, Box 67, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA
E-mail: cioannid@mail.mdanderson.org

Abbreviations: HER-2, HER-2/neu protein; EGF, epidermal growth factor; NDF, neu differentiation factor; Van, sodium orthovanadate; TAL, tumor associated lymphocyte; TPA, triphorbol miristate acetate; OA, okadaic acid; hsp, heat shock protein; TM, transmembrane; LAK, lymphokine activated killer cells; Ub, ubiquitination; (P-Tyr), phosphotyrosine; P-Ser/P-Thr, P-S/P-T phosphoserine/phosphothreonine; ECD, extracellular domain; ICD, intracellular domain; NP, not pulsed

Key words: HER-2 proto-oncogene, HER-2 receptor agonists, tumor associated lymphocytes

Introduction

Immunity to solid tumors usually requires activation of $CD8^+$ tumor-Ag-specific cytotoxic T-lymphocytes (CTL). One pathway of CTL activation originates from antigen presenting cells (APC) that uptake tumor proteins, further process them to peptides, and then represent them to T cells (indirect/cross-priming pathway). Two preconditions for this activation pathway are that tumor cells are damaged and APC are activated (1,2). A second pathway, which has been brought to light only recently (3,4), is the direct activation of T cells by the tumor itself. This pathway appears to be operative under certain conditions, *in vivo*, as demonstrated in model systems (3-6) and may be involved in immunosurveillance and/or induction of split tolerance. A more in-depth understanding of this pathway may help to elucidate mechanisms of immunological ignorance, tolerance to tumors, tumor-induced T cell death (7-9) and may lead to novel approaches that increase the immunogenicity of live tumor cells, prior to APC mobilization.

The poor APC ability of the tumor for priming naïve T cells is believed to be due to reduced expression of MHC-I/II molecules, as well as the lack of costimulatory molecules. However, $CD8^+$ memory/activated cells recognizing epitopes from tumor Ag are frequently found in healthy individuals indicating a priming effect by the tumor Ag when the corresponding protein is expressed at low levels in healthy individuals (10). Tumor cells usually express higher amounts of the tumor protein Ag than normal or hyperplastic cells. Expression of larger amounts of tumor proteins suggests a higher amount of precursor protein for epitope generation, which would lead to preferential loading of peptide epitope derived from these proteins on MHC-I/II. Thus one would expect that *in vivo* the tumor would be more immunogenic than normal cells. The poor immunogenicity of tumor *in vivo* cannot be entirely justified by the low levels of MHC-I and B7 expression by the tumor. The B7 levels may be relevant for priming of naïve T cells but not for re-activation of memory cells, which are less dependent on costimulation. In fact B7-CTLA4 interactions on activated T cells can be inhibitory (9).

Potential mechanisms of Ag ignorance on tumor might be through the increase in Ag half-life by decreasing its rate of degradation or diversion of Ag degradation to pathways which

do not lead to CTL epitope formation. If this is the case, agonists such as EGF and NDF inducing post-translational modifications in the tumor Ag (e.g. phosphorylation, ubiquitination) may affect Ag stability and its degradation.

To address these questions, we investigated as a model the CTL-stimulating ability of breast SKBR3.A2 tumor cells treated with EGF and NDF. SKBR3.A2 cells overexpress HER-2, but express only low levels of MHC-I. The high, stable levels of HER-2 imply a high rate of synthesis and a slow rate of degradation of HER-2 in these cells. An alternative possibility to a higher rate of synthesis is an increased half-life of the tumor Ag of interest by protection via association with chaperones such as heat shock proteins both *in vitro* and *in vivo* (11,12). The effects of EGF ± NDF on tumor sensitivity to CTL lysis and of its CTL activating ability are unknown. HER-2 in these cells is constitutively phosphorylated due to a weak PTK-phosphatase, and its levels of phosphorylation are increased by EGF (13,14). Tyrosine (Tyr)-phosphorylation of HER-2 can be downmodulated by TPA together with the serine-/threonine- (Ser/Thr) phosphatase inhibitor okadaic acid OA; this is accomplished by an increase in Ser/Thr-phosphorylation (OA) (15). Thus, modulation of HER-2 phosphorylation at tyrosine and Ser/Thr by the alternative use of EGF + NDF + Van (E+N+V) and TPA + OA (T+O), provides on approach to address whether phosphorylation agonists induce changes in the ability of tumor cells to stimulate CTL.

We found that pretreatment of SKBR3.A2 cells with E+N+V increased their sensitivity to lysis by HER-2 epitope specific CTL and their ability to induce CTL from non-adherent PBMC and tumor-infiltrating lymphocytes, as compared with control untreated tumor cells. The increased CTL activating ability of E+N+V treated tumors *in vitro* was associated with the absence of a non-ubiquitinated 90 kDa HER-2 intracellular domain (ICD) fragment which was present in control cells, and with the presence of oligo-ubiquitinated forms of HER-2 protein and a 116 kDa C-terminal fragment of HER-2. In contrast, increased sensitivity to lysis in TPA-treated cells was paralleled by a large increase in oligo-ubiquitinated (Ub) HER-2 and the appearance of polyUb-HER-2.

Materials and methods

Reagents, cells and cell lines. HER-2 peptides: F53 (GP2) (654-662): IISAVVGIL (16), C85 (971-979): ELVSEFSRM, and E75 (369-377): KIFGSLALF (17) were prepared by the Synthetic Antigen Laboratory of the M.D. Anderson Cancer Center (16,17). Expression of surface antigens on lymphocytes was determined by FACS analysis, using FITC conjugated mAb specific for CD3, CD4, and CD8. Expression of HLA-A2 on lymphocytes and tumor cells was determined using BB7.2 mAb (ATCC); HER-2 expression was determined using Ab2 or Ab5 mAb; both are specific for the extracellular domain (Oncogene Science, Uniondale, NY). SKBR3.A2 cells transfected with and expressing the gene for HLA-A2 were a kind gift from Drs Martin Cheever and Mary Disis (University of Washington, Seattle, WA). PBMC were isolated from three HLA-A2$^+$ healthy donors (designated as donors 1, 2, and 3). Indicator HLA-A2$^+$ ovarian TAL-1 and -2 were isolated and cultured as described (17).

Immunoprecipitation and immunoblotting. HER-2 protein was immunoprecipitated, detected by immunoblot analysis and analyzed by densitometry as described (18). SKBR3.A2 cells were grown to half-confluence, for 48-72 h, and detached with trypsin. $1x10^6$ cells were resuspended in complete RPMI medium with 5% FCS to reduce the exogenous EGF concentration, and then allowed to adhere and to grow for 24 h. Sixteen hours before the cells were used in experiments, the medium was replaced with complete RPMI + 0.5% FCS (serum starvation). Next, the cells were treated with TPA + OA (T+O) or EGF + NDF ± Van (E+N+V) for 1.5 and 5 h, respectively. Tyrosine phosphorylated HER-2 was detected using the mAb PY-20 (Oncogene Science). Phosphorylation of Thr686 was detected using a P-Thr specific rabbit serum, AP686 (15).

CTL and CTL assays. Indicator E75 specific CTL were obtained by repeated stimulation of plastic non-adherent PBMC of donor 1 with E75 pulsed on autologous monocyte-derived dendritic cells (DC) in complete RPMI medium containing 10% human serum (HS) as we have previously described (19). The percentage of specific tumor lysis was determined from the equation (A-B)/(C-B) x 100. A is the release from tumor in the presence of effectors, B is spontaneous release from tumor cells in the absence of effectors, and C is the maximum ^{51}Cr release. Separate controls of spontaneous and total ^{51}Cr release were made for targets treated with E+N+V or T+O. The experiments were performed in quadruplicate or in triplicate, and the mean ± SD values were calculated from at least two or three separate determinations.

Induction of CTL activity in PBMC and TIL by stimulation with SKBR3.A2 cells. Serum starved SKBR3.A2 cells were treated with E+N+V or T+O for 90 min and then washed and co-cultured with $2x10^6$ plastic non-adherent PBMC at a responder to stimulator ratio of 20:1 in RPMI 1640 medium containing 10% HS (18,19). Control SKBR3.A2 stimulators were treated only with the solvent DMSO, at 0.1% a (final concentration). In some experiments, SKBR3.A2 primed T cells that had been culture in medium containing IL-2, were re-stimulated with SKBR3.A2 at the same S:R ratio and expanded in medium containing IL-2. Ovarian CTL-TAL were rested for 24 h by removing IL-2, then stimulated with SKBR3.A2 cells. To establish the specificity of epitope recognition, we determined the ability of SKBR3.A2 stimulated T cells to recognize the HER-2 peptides E75, GP2, and C85 presented on T2 cells in CTL assays. Specific peptide recognition was determined using T2 cells that were not pulsed with peptide (T2-NP) as a control. Induction of a CTL response to a peptide was considered specific when the difference between the specific lysis of T2 cells pulsed with a particular peptide minus SD was higher than the specific lysis of T2-NP cells plus SD by CTL from the same stimulation group (e.g. E+N+V).

Results

Treatment of SKBR3.A2 cells with EGF + NDF + Van induces high levels of tyrosine phosphorylation of HER-2 protein in

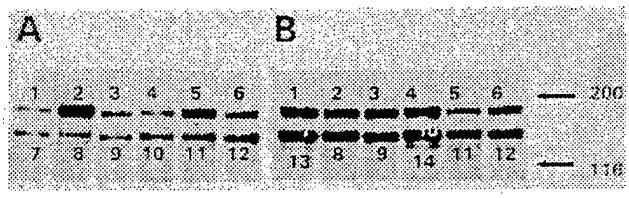


Figure 1. (A), EGF + NDF + Van induced strong and sustained tyrosine phosphorylation of TM-HER-2, but not of intracellular HER-2 (p165). Western blot analysis with PY-20 mAb. Bands 1-3 and 7-9, 90-min treatment; bands 4-6 and 10-12, 5-h treatment. Bands 1, 7, 4 and 10, TPA + OA treatment; bands 2, 8, 5 and 11, E+N+V treatment. Bands 3, 9, 6, 12 control no treatment. Densitometric values (pixel total x 10^{-3}) were: 1, 68.9; 2, 173.1; 3, 69.2; 4, 67.6; 5, 128.1; 6, 85.7; 7, 71.5; 8, 62.3; 9, 61.6; 10, 78.5; 11, 83.8; 12, 108.9. (B), TPA + OA increase T686 phosphorylation. Western blot analysis with AP686 antibody. Positions of all bands are the same as in (A). The same blot was used for reaction with PY-20 and T686 antibodies. Bands 13 and 14 indicate HER-2 of smaller molecular weight which was present only in TPA + OA-treated cells.

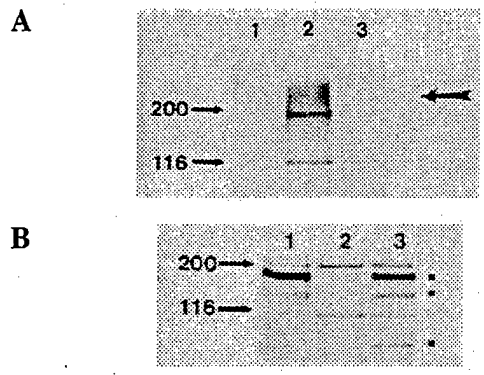


Figure 2. (A), EGF-induced oligo-ubiquitination of HER-2 in SKBR3.A2 cells compared with TPA, which induced both oligo and poly-Ub-HER-2. Western analysis with anti-Ub-Ab of HER-2 immunoprecipitated from SKBR3.A2 cells after: 1, no treatment; 2, T+O treatment; 3, E+N+V treatment. Note the appearance of 200 kDa and >200 kDa bands reactive with anti-Ub-Ab (arrow) in T+O (line 2) and E+N+V (line 3). (B), Analysis of the same blot with anti-HER mAb Ab3. SKBR3.A2 cells were treated with: 1, E+N+V; 2, T+O; 3, control, no treatment. Arrows indicate positions of the 200 and 116 kDa bands ubiquitinated in (A). Dots indicate the positions of the non-ubiquitination bands of 185, 165 and 90 kDa.

the cells. To address whether low concentrations of EGF and NDF induce tyrosine phosphorylation of HER-2, serum starved SKBR3.A2 cells were treated with EGF, NDF, and Van (all at 20 ng/ml) (E+N+V). As a positive control, cells were treated with TPA + OA (both at 5 nM) (T+O). 1.5 h and 5 h later, HER-2 was immunoprecipitated with Ab5 and analyzed in Western blotting with anti-phosphotyrosine (PY-20) and anti-phosphothreonine 686 (AP686) antibodies. The results in Fig. 1A show that E+N+V induced strong tyrosine phosphorylation of the p185 (transmembrane-HER-2), but weak phosphorylation of the intracellular p165 HER-2. These effects were detected at both 1.5 and 5 h E+N+V treatment. The P-Y:P-T 686 ratio was 2-fold higher compared with control, and 3.3-fold higher compared with the T+O treatment. The levels of tyrosine phosphorylation were EGF concentration dependent (data not shown). T+O induced a weaker tyrosine phosphorylation that was observed only at 1.5 h and was mainly due to p165 phosphorylation. Since TPA induced HER-2 poly-ubiquitination (Fig. 2) with consequent mobility shifts, to allow clear detection of the phosphorylated HER-2, TPA was used at a lower concentration (5 ng/ml) than in the ubiquitination and CTL stimulation experiments.

TPA induced a pattern of HER-2 phosphorylation that was distinct from EGF and NDF. The levels of P-T686 in the p185 from T+O treated cells were higher than in control and E+N+V treated cells both at 1.5 and 5 h. In contrast with tyrosine phosphorylation, P-T686 levels were higher in p165 from T+O treated cells at both time points. T+O treated cells also showed a shorter P-T686^{+} chain (~155 kDa) which was not present in the control and E+N+V treated cells. T+O increased T686 phosphorylation but not tyrosine phosphorylation in HER-2 over time compared with control and E+N+V treated cells. These results demonstrated that E+N+V and T+O had distinct effects on tyrosine and T686 phosphorylation levels of HER-2.

EGF + NDF + Van induce weak ubiquitination of HER-2. To address whether growth factors induce ubiquitination and/or cleavage of HER-2, we characterized ubiquitination of HER-2 after E+N+V treatment using TPA-treatment as a positive control. The results in Fig. 2A show that E+N+V induced only weak ubiquitination of the 185 kDa band (detected as a 200 kDa band by the anti-Ub antibody). The 200 kDa band of HER-2 in both T+O and E+N+V treated cells most likely represented an oligo-ubiquitinated product [(Ub = 9 kDa); 185+2x9=203 kDa]. T+O induced not only oligo-ubiquitination (the 200 kDa band) but also a strong poly-ubiquinated band of high molecular weight. Significantly higher amounts of the 200 kDa oligo-Ub-HER-2 were present in T+O treated cells than in E+N+V treated cells. An additional oligo-Ub-HER-2 band of 116 kDa was present in larger amounts in T+O treated than E+N+V treated cells, but was absent from control untreated cells.

Incubation of the same blots with Ab3 mAb revealed a similar pattern of immunoreactive bands (185, 165 and 116 kDa) in control and E+N+V treated cells (Fig. 2B, line 3 vs. line 1). T+O treated SKBR3.A2 cells lacked both the 185 and 165 kDa bands, confirming that in these cells most HER-2 was ubiquitinated as a whole molecule (Fig. 2B line 2). An additional non-Ub band of 90 kDa HER-2 was present only in the control cells (Fig. 2B, line 3). The decrease in the levels of this band was dependent on: a) the EGF concentration since it was present in cells treated with lower amounts (≤2 ng) of EGF and; b) the presence of OA, since it was present in cells treated with low concentrations of TPA (data not shown). This band represents a large fragment of the intracellular HER-2 domain (ICD), as indicated by the fact that it reacted with Ab3, which recognizes a C-terminal epitope on HER-2. This epitope would have not been recognized by Ab3 if the 90 kDa band was part of the HER-2 ECD. It is possible that the 116 kDa band in E+N+V treated and T+O treated cells in Fig. 2A represent an oligo-ubiquitinated form of the 90 kDa HER-2 fragment.

These results indicate that E+N+V induced oligo-ubiquitination only of a small part of HER-2. E+N+V and

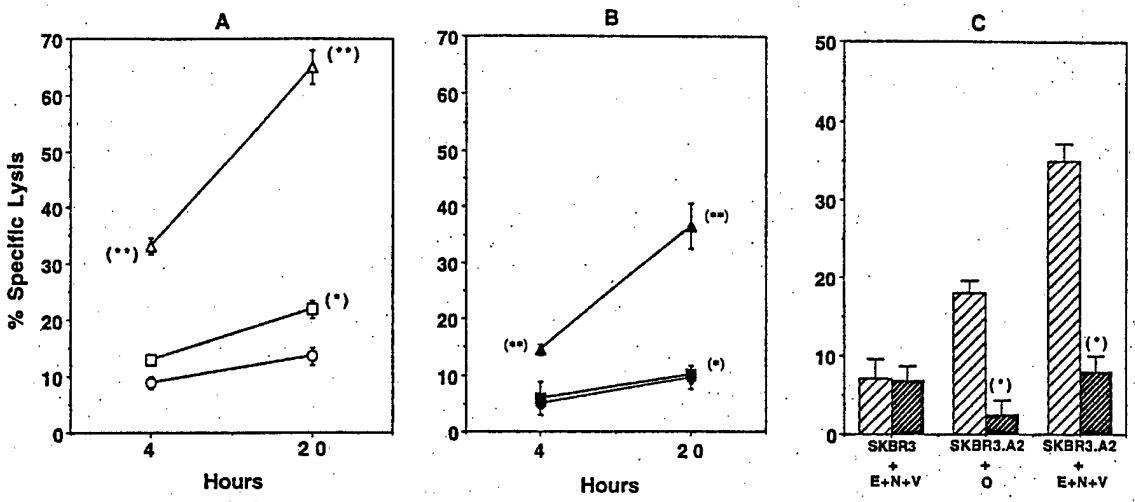


Figure 3. (A) Sensitivity to lysis of SKBR3.A2 by OVA-TAL-1. Targets were: (0) control untreated tumor cells; tumor treated with (E+N+V) and (Δ) tumor treated with T+O. Results indicate percent specific lysis of targets in a 4 and a 20-h CTL assay. *, **Levels of lysis of T+O treated SKBR3 were significantly higher than of control non-treated cells ($p<0.05$) and $p<0.01$, respectively. (B), Residual SKBR3.A2 cells lysis in the presence of α-HLA-A2 mAb (BB7.2) added to the targets prior to addition of effectors. •, Control untreated; ▪, E+N+V treated; Δ, T+O treated SKBR3.A2; *SKBR3.A2 lysis in the presence of BB7.2 was significantly lower ($p<0.05$) than lysis in the absence of BB7.2 shown in (A) (□). **Lysis of T+O treated SKBR3.A2 was significantly lower in the presence of BB7.2 than in the absence of BB7.2 shown in (A) (▲). (C), E+N+V treatment increased sensitivity to CTL lysis of SKBR3.A2 cells but not of control SKBR3 cells. Lysis of E+N+V treated SKBR3.A2 cells was inhibited by 'cold' T2 pulsed with E75 at comparable levels with lysis of SKBR3.A2 cells. SKBR3 + E+N+V indicate parental control HLA-A2- targets treated with E+N+V. *Significant inhibition of lysis ($p<0.02$). Cold-targets: white striped bar, T2-NP; black striped bar, T2-E75.

T+O treatments induced different molecular species of HER-2 in SKBR3.A2 cells. E+N+V treated cells differed from control cells by the absence of the non-Ub 90 kDa ICD (Fig. 2B) and the presence of oligo-Ub-116 kDa and oligo-Ub-200 kDa HER-2 (Fig. 2A). The absence of the 90 kDa ICD from both E+N+V and T+O treated cells suggested that either the HER-2 molecule was not degraded to the 90 kDa ICD, or that this fragment was degraded faster than in control cells and therefore could not be detected. Alternatively, the oligo-Ub-116 kDa HER-2 may contain a part of the 90 kDa fragment (90+3x9=117 kDa). Other HER-2 bands were expressed at low levels in all samples as determined by densitometry (<10% variability) (data not shown), and there were only small quantitative differences between cells.

Since ubiquitination of surface proteins is associated with their downmodulation, we determined the levels of surface expression of HER-2. Since the amounts of E+N used could have different effects on cells expressing high vs. low levels of HER-2, we determined the levels of HER-2 expression in both SKBR3.A2 (HER-2^{hi}) and MB231 (HER-2^{lo}) cells, after treatment with E+N+V and T+O. E+N+V did not affect surface HER-2 expression on these cells, as compared with control untreated cells. The surface HER-2 levels declined by 30% in the cells treated with T+O as indicated by the MCF values shown in parenthesis SKBR3.A2: control (107), T+O (87), E+V (106); similarly: MB231, control (43), T+O (32), E+V (42) confirming that TPA-induced ubiquitination was directed to the surface receptor.

Treatment of SKBR3.A2 cells with E+N+V and T+O increase their recognition by ovarian CTL-TIL. To address whether growth factor treatment affects tumor antigenicity, we performed cytolysis experiments. The results in Fig. 3A show that E+N+V induced a small increase in the lysis of SKBR3.A2 cells by ovarian CTL-TAL-1 (OVA-TAL-1) in the 4-h CTL assay (45%) compared with control activated tumors. In contrast, T+O induced a significant increase in the lysis of tumor cells (3.74-fold). The level of lysis increased over time in the assay (20 h). To determine whether the changes in lysis of tumor cells by CTL effectors are due to presentation of MHC-I-associated epitopes, α-HLA-2 mAb (BB7.2) was added to the assays performed in parallel. The inhibition was higher for the E+N+V and T+O treated cells than for the control untreated cells (Fig. 3B). In fact, when the residual lysis (measured in the presence of BB7.2) is subtracted from the lysis of targets where BB7.2 was absent, the increase in lysis for the E+N+V and T+O treated targets was even higher than of control cells (by 80 and 482%). In the 20-h CTL assay BB7.2 mAb was more effective in inhibiting the lysis of E+N+V treated cells (by 54%) compared with untreated SKBR3.A2 cells (30%). Similarly subtraction of the residual lysis indicated a higher recognition of E+N+V and T+O treated cells. These results indicate that T+O induced poly-Ub leads to a high and rapid increase in CTL epitope presentation compared with oligo-Ub induced by E+N+V. To confirm these results for the HER-2 CTL epitope E75, we performed cold-target inhibition experiments (Fig. 3C). We used E75-specific CTL as effector (19) and E+N+V treated and untreated SKBR3.A2 cells as targets. The results show that E+N+V increased the sensitivity of SKBR3.A2 to lysis by 90% (from 18.0% in control to 35.9% in E+N+V treated cells). E75 pulsed T2 cells inhibited SKBR3.A2 lysis to similar extents (86 vs. 78% inhibition in both control and treated group) indicating that the increase in lysis was not due to increase of lysability

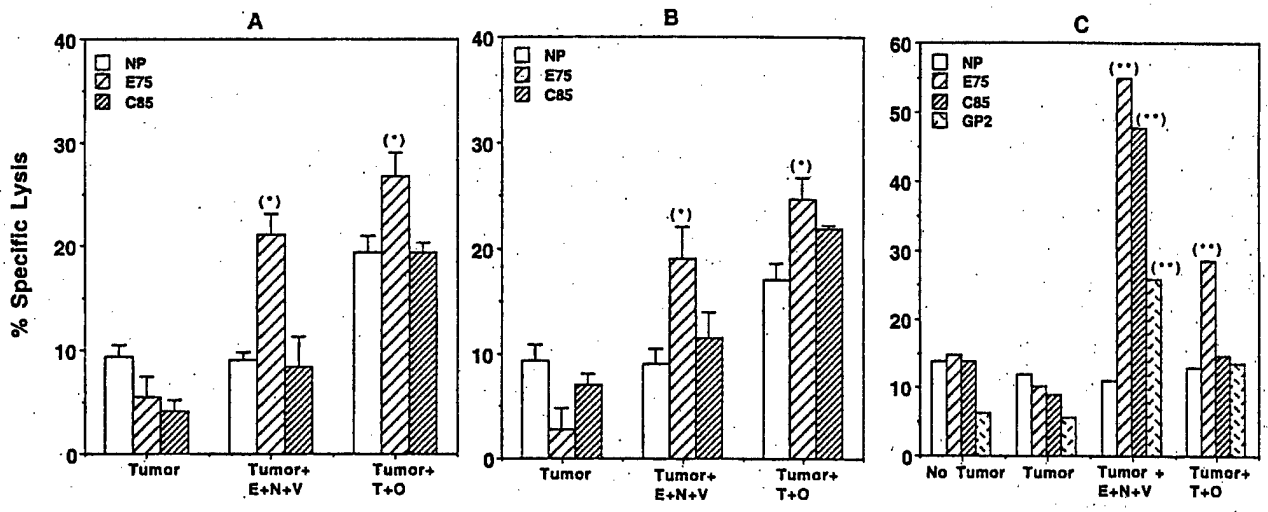


Figure 4. Stimulation of CTL activity by SKBR3.A2 treated with E+N+V. (A), Donor 2; 4-h CTL assay. (B), Donor 3, 8-h CTL assay. (C), OVA-TAL-2, 4-h CTL assay. Non-adherent PBMC (A and B) and OVA-TAL (C) were either not co-cultured with tumor (no tumor), or co-cultured with SKBR3.A2 cells (tumor) or co-cultured with SKBR3.A2 cells treated with E+N+V (tumor + E+N+V) or with T+O (tumor + T+O). % specific lysis indicate the specific lysis of T2 cells pulsed with peptides, E75, C85 and GP2 as well as the background lysis of T2 not pulsed with peptide (T2-NP), as shown in the figure. *Significantly higher lysis of T2-E75 than of T2-NP ($p<0.05$) (A and B). **Significantly higher lysis ($p<0.01$) of T2-E75, T2-C85 and T2-GP2 compared with targets T2-NP (C).

of tumor, but rather to higher E75 presentation. Lysis of control HLA-A2$^-$ SKBR3 cells treated with E+N+V by E75-specific CTL was borderline and was not inhibited by T2-E75.

E+N+V increased CTL-stimulating ability of SKBR3.A2 cells. To address whether E+N+V enhanced the ability of SKBR3.A2 cells to induce CTL, we used as responders both plastic non-adherent PBMC from healthy donors and TAL from ovarian cancer patients. PBMC contain mainly naïve T cells together with a small number of previously activated and/or partially tolerized CTL, while TAL contain mainly activated CD8$^+$ T cells. We tested recognition of the defined HER-2 CTL epitopes, E75, C85, and GP2. The results in Fig. 4A show that priming of plastic non-adherent PBMC from donor 2 with SKBR3.A2 cells treated with E+N+V led to CTL which recognized E75 well, but C85 only marginally. The CTL-stimulating effect of SKBR3.A2 cells treated with T+O was much weaker. Control, untreated SKBR3.A2 cells were ineffective in inducing CTL at priming. This experiment was repeated using as effectors plastic non-adherent PBMC from donor 3, stimulated with SKBR3.A2 cells and the results were confirmed (Fig. 4B). These results indicate that E+N+V treated SKBR3.A2 cells are better stimulators for priming of CTL activity from PBMC than their untreated or T+O treated counterparts.

To address whether EGF and NDF-treated tumor cells had similar effects on activated T cells, we tested their stimulatory ability for activation of CTL function of TIL. TAL lack conventional APC, thus in this system only the tumor can function as APC. We selected an ovarian TAL line (OVA-TAL-2) which at the time of stimulation showed non-specific recognition of all CTL epitopes tested (E75, C85, and GP2) (Fig. 3, group no tumor). This line was selected because 3 weeks earlier it showed specific recognition of all three CTL epitopes (data not shown). Thus at the time of tumor stimulation, either the number of effectors was below the numbers required to induce detectable specific lysis or the effectors had lost a significant part of their lytic activity. CTL from the SKBR3.A2$^+$ E+N+V stimulation group showed a >4-fold increase in recognition of E75 and C85 compared with unstimulated CTL. Only E+N+V treated SKBR3.A2 cells were able to activate CTL recognizing C85 and GP2, suggesting differences in epitope presentation by E+N+V and T+O treatments. These results demonstrate that EGF + NDF treatment of tumor cells can enhance their ability to activate CTL in both PBMC and TAL.

Discussion

In this report, we provide evidence that treatment of SKBR3.A2 cells with EGF and NDF lead to an increased ability of stimulate CTL. This conclusion is supported by results that EGF + NDF treated SKBR3.A2 cells directly activated TAL. CTL-TAL are long-term cultured T cell lines of CD4$^+$ and CD8$^+$ phenotype. Macrophages, B cells and DC are not present in cultured TAL. These effects were not observed with control tumor cells. This conclusion is also supported by results obtained with SKBR3.A2 treated with TPA + OA.

EGF and NDF act as phosphorylation agonists and activate a large number of signaling pathways. While the precise effects of EGF and NDF in inducing SKBR3.A2 activating ability for CTL remain to be determined, we demonstrate that EGF and NDF have specific effects on HER-2 phosphorylation and ubiquitination, which are distinct from the effects of TPA + OA. First, the effects of EGF + NDF in inducing oligo-Ub-HER-2 appeared quite specific, since TPA + OA induced mainly poly-Ub-HER-2. Second, Ub-HER-2 patterns reflected distinct HER-2 phosphorylation patterns. EGF and NDF induced high levels

of P-Tyr in HER-2, but low levels of P-T686. TPA + OA increased P-T686 robustly but P-Tyr only modestly. Our results suggest that oligo-Ub-HER-2 induced by EGF and NDF may be the consequence of its modification by tyrosine phosphorylation (20). T+O favored the rapid formation of poly-Ub-HER-2 (whole molecule). Thus the CTL activating ability of EGF and NDF-treated tumor cells compared with control cells may be due to differences in the rate of formation of epitope precursors and of epitopes themselves, which may be processed by different systems with different kinetics (21).

T+O induced poly-Ub-HER-2 may lead to higher initial epitope presentation, as illustrated by the higher levels of lysis of TPA + OA-treated SKBR3.A2 compared with EGF and NDF-treated cells. In contrast, degradation of the oligo-Ub$^+$ HER-2 or of P-Tyr-HER-2 by cytosolic proteases (and/or the proteasome) may provide a smaller but stable increase in epitope production and presentation over time (22,23). This may avoid CTL overstimulation. Ongoing studies are investigating the significance of the C-terminal tyrosine phosphorylation (Y1248) in modulating the antigenicity and immunogenicity of HER-2 for breast tumor associated lymphocytes (Castilleja *et al*, unpublished data).

Transmembrane HER-2 mediate its proliferative effects by association with EGF-R and HER-3, after phosphorylation in response to agonists such as EGF and NDF (24). Excess signaling complexes are downmodulated, ubiquitinated, and further degraded by both cytosolic and lysosomal proteases (14). HER-2 aggregation, downmodulation, and degradation are modulated by EGF. Low EGF concentrations promote signaling, while high EGF concentrations promote down-modulation. The EGF effects are modulated by feed-back activation of tyrosine phosphatases which show variable levels of activity in various tumor cells (25).

The experimental conditions in this study were chosen to avoid downmodulation of HER-2 (and implicitly termination of signaling by EGF + NDF), by high EGF concentrations plus culture in high serum and to inhibit tyrosine phosphatases using Van, in an attempt to mimic the conditions corresponding to a surface receptor continuously transducing phosphorylation signals. The hypothesis that the antigenicity and immunogenicity of SKBR3.A2 were dependent on the EGF and TPA concentrations is supported by the fact that low concentrations of EGF (1-2 ng/ml) and TPA (1-5 ng/ml) were ineffective in increasing CTL activating ability by SKBR3.A2, while high concentrations of Van had inhibitory effects on SKBR3.A2 antigenicity. The latter effects were reversed by OA. In fact, low EGF and TPA concentrations, in the absence of Van and of OA, respectively, were associated with the presence of the 90 kDa ICD in immunoprecipitates (Castilleja *et al*, unpublished data).

At this time, we cannot distinguish whether the CTL-stimulating effects of E+N+V treated cells are due to: a) stimulation of cellular synthesis with consequent increases in *de novo* synthesized HER-2; b) increased cytosolic protease activity or; c) stable tyrosine-phosphorylated HER-2. Our findings raise the possibility that the EGF and NDF effects may endow tumor cells with the ability to modulate lymphocyte activation. One possibility is that in the absence of EGF, tyrosine phosphorylation of TM-HER-2 is weak and unstable, thus due its long half-life it will produce small amounts of epitopes. EGF will induce tyrosine phosphorylation of TM-HER-2. TM-HER-2 is oligo-ubiquitinated and its faster degradation may provide epitopes for CTL activation in premalignant states such as hyperplasia or DCIS. High EGF concentrations increase formation of HER-2: EGF-R:HER-3 complexes, which, after internalization may either, recycle or are degraded by lysosomal proteases. This is less likely to increase CTL epitope presentation. The EGF effects are controlled by the activity of tyrosine phosphatases and protein (Ser/Thr) phosphatases, whose level of activation vary among various tumor cells. Identification of modulation of CTL activation by tumor treatment with growth factors and tyrosine phosphatase inhibitors may allow development of novel therapies directed to drug resistant tumors and design of better HER-2 based immunogens for cancer vaccination.

Acknowledgements

This study was supported in part by the Core Grant CA-16662 and the DOD Grant 17-97-I-7098 and the Woman's Fund for HER.

References

1. Huang AY, Bruce AT, Pardoll DM and Levitsky HI: Does B7-1 expression confer antigen-presenting cell capacity to tumors *in vivo*? J Exp Med 183: 769-776, 1996.
2. Ronchetti A, Rovere P, Iezzi G, Galati G, Heltai S, Protti MP, Garancini MP, Manfredi AA, Rugarli C and Bellone M: Immunogenicity of apoptotic cells *in vivo*: role of antigen load, antigen-presenting cells and cytokines. J Immunol 163: 130-136, 1999.
3. Armstrong TD, Clements VK and Ostrand-Rosenberg S: Class II-transfected tumor cells directly present endogenous antigen to CD4$^+$ T cells *in vitro* and are APCs for tumor-encoded antigens *in vivo*. J Immunol 21: 218-224, 1998.
4. Armstrong TD, Clements VK and Ostrand-Rosenberg S: MHC class II-transfected tumor cells directly present antigen to tumor-specific CD4$^+$ T lymphocytes. J Immunol 160: 661-666, 1998.
5. Seung LP, Rowley DA and Schreiber DP: Synergy between T cell immunity and inhibition of paracrine stimulation causes tumor rejection. Proc Natl Acad Sci USA 92: 6254-6258, 1995.
6. Liu Y, Wang H, Zhao J, Ma J, Wei L, Wu S, Xie T, Shen F, Trojan J, Habib N, Anthony DD, Wu M and Guo Y: Enhancement of immunogenicity of tumor cells by cotransfection with genes encloding antisense insulin-like growth factor-1 and B7.1 molecules. Cancer Gene Ther 7: 456-465, 2000.
7. Chappell DB and Restifo NP: T cell-tumor cell: a fatal interaction? Cancer Immunol Immunother 47: 65-71, 1998.
8. Ocsenbein AF, Klenerman P, Karrer U, Ludewig B, Pericin M, Hengartner H and Zinkernagel RM: Immune surveillance against a solid tumor fails because of immunological ignorance. Proc Natl Acad Sci USA 96: 2233-2238, 1999.
9. Shrikant P, Khoruts A and Mescher MF: CTLA-4 blockade reverses CD8$^+$ T cell tolerance to tumor by a CD4$^+$ T cell-and IL-2-dependent mechanism. Immunity 11: 483-493, 1999.
10. Lee TV, Anderson BW, Peoples GE, Castilleja A, Murray JL, Gershenson DM and Ioannides CG: Identification of activated tumor antigen-reactive CD8$^+$ cells in healthy individuals. Oncol Rep 7: 455-466, 2000.
11. Pasleau F, Grooteclaes M and Gol-Winkler R: Expression of the c-erb2 gene in the BT474 human mammary tumor cell line: measurement of c-erbB2 mRNA half-life. Oncogene 8: 849-854, 1993.
12. Chavany C, Mimnaugh E, Miller P, Britton R, Nguyen P, Trepel J, Whitesell L, Schnur R, Moyer JD and Neckers L: p185erbB2 binds to grp94 *in vivo*. J Biol Chem 271: 4974-4977, 1996.
13. Peles E, Ben-Levy R, Tzahar E, Liu N, Wen D and Yarden Y: Cell-type specific interaction of Neu differentiation factor (NDF/Her-2) suggests complex. EMBO J 13: 961-971, 1993.

14. Gilboa L, Ben-Levy R, Yarden Y and Henis YI: Roles for a cytoplasmic tyrosine and tyrosine kinase activity in the interactions of Neu receptors with coated pits. J Biol Chem 270: 7061-7067, 1995.
15. Quyang X, Zhang H, Huang GC and Epstein R: Human cancer cells exhibit PKC-dependent c-erb-2 transmodulation that correlates with phosphatase sensitivity and kinase activity. J Biol Chem 271: 21786-21792, 1996.
16. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I and Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci USA 92: 432-436, 1995.
17. Fisk B, Blevins TL, Wharton JT and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. J Exp Med 181: 2109-2117, 1995.
18. Castilleja A, Ward NE, O'Brian CA, Swearingén B II, Swan E, Gillogly MA, Murray JL, Kudelka AP, Gershenson DM and Ioannides CG: Accelerated HER-2 degradation enhances ovarian tumor recognition by CTL. Implications for tumor immunogenicity. Mol Cell Biochem 217: 21-33, 2001.
19. Anderson BW, Peoples GE, Murray JL, Gillogly MA, Gershenson DM and Ioannides CG: Peptide priming of cytolytic activity to HER-2 epitope 369-377 in healthy individuals. Clin Cancer Res 6: 4192-4200, 2000.
20. Hicke L: Ubiquition-dependent internalization and down-regulation of plasma membrane proteins. FASEB J 11: 1215-1226, 1997.
21. Grant EP, Michalek MT, Goldberg AL and Rock KL: Rate of antigen degradation by the ubiquitin-proteasome pathway influences MHC class I presentation. J Immunol 155: 3750-3758, 1995.
22. Mimnaugh EG, Chavany C and Neckers L: Polyubiquitination and proteasomal degradation of the p185 receptor protein-tyrosine kinase induced by gelanamycin. J Biol Chem 271: 22796-22801, 1996.
23. Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdel JW and Bennink JR: Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 404: 770-774, 2000.
24. Yewell JW, Norbury CC and Bennink JR: Mechanisms of exogenous antigen presentation by MHC class I molecules *in vitro* and *in vivo*: implications for generating $CD8^+$ T cell responses to infectious agents, tumors, transplants and vaccines. Adv Immunol 73: 1-77, 1999.
25. Wells A, Welsh JB, Lazar CS, Wiley HS, Gill GN and Rosenfeld MG: Ligand-induced transformation by a non-internalizing epidermal growth factor receptor. Science 247: 962-964, 1994.

Molecular and Cellular Biochemistry **217:** 21–33, 2001.

Accelerated HER-2 degradation enhances ovarian tumor recognition by CTL. Implications for tumor immunogenicity

Agapito Castilleja,[1] Nancy E. Ward,[2] Catherine A. O'Brian,[2] Bruce Swearingen II,[1] Eric Swan,[1] Michael A. Gillogly,[3] James L. Murray,[3] Andrzej P. Kudelka,[4] David M. Gershenson[1] and Constantin G. Ioannides[1, 5]

Departments of [1]Gynecologic Oncology; [2]Cancer Biology; [3]Bioimmunotherapy, [4]Gynecologic Medical Oncology; [5]Immunology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA

Received 3 April 2000; accepted 6 October 2000

Abstract

We investigated the ubiquitination and degradation of a tumor antigen, the HER-2/neu (HER-2) protooncogene product which is overexpressed in epithelial cancers. HER-2 degradation was investigated in the ovarian tumor line, SKOV3.A2, that constitutively overexpressed long-life HER-2. We used as agonist geldanamycin (GA), which initiated downmodulation of HER-2 from the cell surface. HER-2 was polyubiquitinated and degraded faster in the presence than in the absence of GA. GA did not decrease HLA-A2 expression. Presentation of the immunodominant cytotoxic T lymphocyte (CTL) epitope, E75 (369–377) from SKOV.A2 was inhibited by proteasome inhibitors, such as LLnL but was enhanced by cysteine protease inhibitors such as E64, indicating that both the proteasome and cysteine proteases are involved in epitope formation but have different effects. Enhanced tumor recognition was not an immediate or early effect of GA treatment, but was evident after 20 h of GA treatment. In contrast, 20 h GA treatment did not increase tumor sensitivity to LAK cell lysis. Twenty hour GA-treated SKOV3.A2 cells expressed an unstable HER-2 protein synthesized in the presence of GA, of faster electrophoretic mobility than control HER-2. This suggested that the newly synthesized HER-2 in the presence of GA was the main source of epitopes recognized by CTL. Twenty hour GA-treated SKOV3.A2 cells were better inducers of CTL activity directed to a number of HER-2 CTL epitopes, in peripheral blood mononuclear cells compared with control untreated SKOV3.A2 cells. Thus, induction of HER-2 protein instability enhanced the sensitivity of tumor for CTL lysis. Increased HER-2 CTL epitopes presentation may have implications for overcoming the poor immuno-genicity of human tumors, and design of epitope precursors for cancer vaccination. (Mol Cell Biochem **217**: 21–33, 2001)

Key words: CTL, HER-2 recognition, degradation, ubiquitination, geldanamycin

Abbreviations: CTL - cytotoxic T lymphocytes; HER-2 – HER-2/neu oncogene; GA – geldanamycin; PA – proteasome; LC – lactacystin; hsp – heat shock protein; grp94 – glucose-regulated protein; LC – lactacystin; Ub – ubiquitin; Ub-HER-2 – ubiquitinated HER-2; (Ub)-HER-2 – non-ubiquinated HER-2; Act D – actinomycin D; CHX – cycloheximide; MCF – mean channel fluorescence; EGF – epidermal growth factor; NDF – neu differentiation factor; TAL – tumor associated lymphocytes

Address for offprints: C.G. Ioannides, Department of Gynecologic Oncology, Box 67, The University of Texas, M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

Introduction

Cytotoxic T lymphocytes (CTL) were demonstrated to prevent or eradicate experimental tumors in animal models [1]. In human cancers, these CTL usually fail to control tumor growth. A number of reasons for these failures have been reported. Some appear to be unrelated to tumor Ag expression (i.e. defective lymphocyte homing, tumor induced apoptosis) [2–4]. Others appear to be directly and/or indirectly related to the presence of Ag on the tumor and the poor presentation and immunogenicity of the latter [5–7]. These results suggested that for solid tumor, that express a particular tumor Ag, an increase either in density or in persistence of CTL epitopes on MHC-I may increase the sensitivity of tumor to CTL lysis. This may provide an approach to control tumor escape. Enhancing CTL epitope presentation may be beneficial not only for reversing tumor escape but also for design of epitope precursors which can present the epitope at high levels. These precursors can be incorporated in genetically engineered vaccines.

One approach to this question is to direct the tumor Ag for degradation by the MHC-I processing pathways [8–11] and to enhance its rate of degradation. This is based on the expectation that degradation with accelerated kinetics will lead to higher density of the CTL epitopes of interest on MHC-I. This will result in increased tumor sensitivity to lysis because high density Ag presentation will be able to activate low-affinity CTL. The MHC-I processing pathway requires ubiquitination as a precondition for targeting of folded proteins for cytosolic degradation by the 26S proteasome (PA) [12, 13]. Non-proteasomal cytosolic proteases such as cysteine proteases were recently reported to play a significant role in N-terminus epitope precursor liberation from minigene expressed foreign Ag [14–16].

There is little information on the modalities of directing transmembrane self-proteins for MHC-I processing pathways and the effects of this targeting on tumor Ag recognition. This paucity of information is compounded by the facts that (a) self-proteins with long half-life should expose less protease sensitive sites than imported foreign proteins [11], (b) transmembrane self-proteins such as HER-2 are protected from degradation by forming complexes with stress proteins, e.g. grp94 and (c) the specificity and activity of cytosolic and endoplasmic reticulum proteases in tumors and professional APC are not identical [17].

To address these questions, we focused on the HER-2 protooncogene product. The majority of HER-2 protein is located at the transmembrane (TM) on both tumor and normal cells, and it is characterized by a long half-life (~ 7 h) [18, 19]. To induce HER-2 degradation through the MHC-I pathway, we used geldanamycin (GA). GA was originally considered a specific receptor tyrosine kinase (RTK) inhibitor [19]. Recent studies have shown that GA mediated its inhibitory effects by binding strongly to stress proteins such as heat shock protein (hsp90) or glucose regulated protein (grp94), which form complexes with HER-2, and inhibits indirectly by destabilizing stress protein complexed kinases [20]. GA does not inhibit HER-2 mRNA and protein synthesis in tumor cells, but by dissociating the HER-2:grp94 complex, reduces the protein half-life [20, 21]. GA treatment of cells increased the rate of ubiquitination of existent HER-2 with consequent faster proteasomal degradation. HER-2 synthesized in the presence of GA is also unstable and does not accumulate in tumor cells [19, 20]. It is unknown whether induction of polyubiquitination at a faster rate of existent long-life transmembrane HER-2 enhances CTL epitope presentation. Furthermore, since HER-2 synthesized in the presence of GA is unstable, recognition of tumor cells also expressing this unstable protein may show enhanced epitope presentation.

To identify which HER-2 chain is a better source of epitope, we used as targets the ovarian SKOV3.A2 cells. SKOV3.A2 express high and stable levels of HER-2 and of transfected HLA-A2 [22]. In these cells, HER-2 is not autophosphorylated at Y1248 and the other RTK sites as in SKBR3 cells due to the constitutive presence of an extremely active protein tyrosine phosphatase (PTP), but it is constitutively phosphorylated at Thr686 by protein kinase C [22, 23]. As a consequence, SKOV3.A2 proliferation is less dependent on mitogenic signaling by tyrosine phosphorylated HER-2 and as a result less susceptible to inhibition by GA. Thus, decreased HER-2 protein expression following GA-treatment could not be attributed to GA induced growth arrest while the effects of GA on epitope presentation will not be masked by inhibition of HER-2 protein synthesis.

We found that GA rapidly downmodulated transmembrane HER-2 and accelerated its ubiquitination and degradation. GA did not increase HER-2 CTL epitope presentation during the first 3–5 h of treatment. Significantly increased presentation of the epitope E75 was detected after 20 h GA treatment. This coincided with expression by tumor cells of lower levels of HER-2 of faster electrophoretic mobility than the HER-2 protein present in control cells. These findings are novel and suggest that the unstable HER-2 synthesized in the presence of GA participate in CTL epitope formation.

Materials and methods

Reagents, synthetic peptides, monoclonal antibodies and immunofluorescence

GA was obtained from Dr. Jill Johnson, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI. GA was dissolved in dimethylsulfoxide (DMSO) and used for cell treatment at various concentrations ranging from 50 nM to 10 μM. In all experiments, the DMSO concentra-

tion in culture did not exceed 0.1%. Lactacystin (LC) was obtained from Professor E.J. Corey, Harvard University, USA. E64 (cysteine protease inhibitor) and LLnL (proteasome inhibitor), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HER-2 peptides: E91 (5–13) ALCRWGLLL, D113 (48–56) HLYQGCQVV, E89 (851–859), VLVK SPNHV C85 (971–979) ELVS EFS RM, E75: KIFGSLALF (369–377), E71: QLLMPYGLLL (799–807), F57 (439–447) IHLNGSAYSL and modified control MUC-1 peptide D125: GLTSKDTRV (16–24) were prepared by the Synthetic Antigen Laboratory at the U.T. M.D. Anderson Cancer Center, TX, USA [22]. Expression of surface antigens on lymphocytes was determined by FACS analysis, using FITC conjugated mAb specific for CD3, CD4, CD8, (Ortho Diagnostic, Raston, NJ, USA). Expression of HLA-A2 was determined using BB7.2 mAb (ATCC); HER-2 expression was determined using Ab2 mAb or Ab5 mAb specific for the extracellular domain and Ab3 specific for the intracellular domain (Oncogene Science, Uniondale, NY, USA). Expression of CD13, CD14, CD80 (B7.1), CD86 (B7.2), ICAM-1 (CD54), CD40, and MHC-class II on tumor and dendritic cells was detected using the corresponding specific mAb FITC or phycoerythrin conjugated (Beckton-Dickinson).

Cells and cell lines

The ovarian tumor line SKOV3 transfected with and expressing the gene for HLA-A2 has been previously described [22]. PBMC were isolated from two HLA-A2$^+$ healthy donors, (designated as donor 1 and donor 2). Ovarian TAL were isolated and cultured as described [22].

Immunoprecipitation and immunoblotting

HER-2 protein was precipitated from SKOV3 cells and detected as described [19] with several modifications [24]. SKOV3.A2 cells were grown to half-confluence for 48–72 h, detached with trypsin, resuspended in complete RPMI medium with 5% FCS to reduce the exogenous EGF concentration, re-plated at a concentration of 2×10^6 cells/ml, and treated with GA and/or LC for different time intervals. These conditions assured that tumor cells were neither growth arrested nor serum starved. In brief, SKOV3.A2 cells were washed with ice-cold PBS, resuspended in lysis buffer (20 mM Tris.HCl, pH 7.5) containing 55 mM EDTA, 1% Triton, 5 mM EGTA, 15 mM 2-mercaptoethanol, and protease inhibitors as follows: 20 ng/ml serum trypsin inhibitor (Sigma), 10 ng/ml leupeptin (Boehringer), and 0.25 mM phenylmethylsulfonyl fluoride (PMSF). After disruption using 30–40 strokes with the Dounce homogenizer, membranes were allowed to solubilize for an additional 1 h in the lysis buffer. The lysate was cleared of debris by centrifugation at 10,000 g for 15 min. Protein concentration of the cleared cell lysate was determined by the BioRad Method and adjusted before precipitation to 1 mg/ml [19, 24]. HER-2 was immunoprecipitated with 2 μg/ml of anti-neu, Ab5 followed by 5 μg/ml of rabbit-anti-mouse IgG (DAKO, Dakopatts, Denmark). Immune complexes were collected on Protein-A-Sepharose beads (Sigma) eluted by boiling in SDS loading buffer, fractionated on 7.5% SDS-PAGE, and transferred onto nitrocellulose membranes (Bio-Rad). HER-2 was detected on blots using Ab3, while ubiquitinated HER-2 (Ub$^+$-HER-2) was detected with anti-ubiquitin (Ub) polyclonal antiserum (Sigma) followed by appropriate horseradish-peroxidase linked secondary antibodies and visualized by chemiluminescence [19–24]. For densitometry, HER-2 peaks in Western blots from the anti-neu experiments were scanned using a Precisim™ Densitometer (Molecular Dynamics, Sunnyvale, CA, USA) and quantitated using the program Image Quant™.

Indicator E75-specific CTL

Indicator E75 specific CTL were obtained by repeated stimulation of plastic non-adherent PBMC of donors 1 and 2 with E75 pulsed on autologous monocyte-derived dendritic cells (DC) [26] in complete RPMI medium containing 10% human serum (HS) as we described followed by expansion in complete RPMI medium containing IL-2. Autologous DC of CD14$^+$, CD13$^+$, CD80$^+$, CD86$^+$, CD40$^+$, MHC-I$^+$, and MHC-II$^+$ phenotype were obtained by culturing the plastic adherent cells of PBMC for 5–6 days in 1000 U/ml GM-CSF (Immunex Corp., Seattle, WA, USA, specific activity 1.25×10^7 CFU/250 mg) and 500 U/ml of IL-4 (Biosource International, specific activity 2×10^6). Specificity of recognition was determined 7 days after the last stimulation by comparing lysis of T2 cells pulsed with E75 (at 25 μg/ml) vs. no peptide (NP) or control peptides such as E71 or D125 pulsed at the same concentration. DC-E75 primed PBMC were tested for recognition of E75 presented by T2 cells and endogenous epitope by recognition of the indicator SKOV3.A2 HER-2$^+$ tumor line. Some healthy donors responded to peptide stimulations with E75 for CTL induction [26]. Specificity for E75 presented by the tumor was determined in cold-target inhibition assays of lysis of SKOV3.A2 cells by T2 cells pulsed with E75, E71, D125, or NP [22].

CTL assays

To determine the role of the proteasome and cytosolic proteases on epitope formation, all inhibitors at the highest concentrations previously reported to inhibit Ag presentation [18, 19]: LLnL (50 μM) and E64 (50 μM) were used to pre-treat

the targets for 2 h before labeling, during labeling, before addition of effectors, and they were further maintained in the assay at suboptimal concentrations, (25 μM) to delay the reversal of their effects as described [13, 17–19, 27]. Percentage of specific tumor lysis was determined from the equation (A–B)/(C-B) × 100. A is the release from tumor in the presence of effectors, B is spontaneous release from tumor cells in the absence of effectors, and C is the maximum ^{51}Cr release. Separate controls of specific and total ^{51}Cr release were made for targets treated with inhibitors. The experiments were performed in triplicate, and the mean ± S.D. values were calculated from at least 2 separate experiments.

Induction of CTL activity in PBMC by stimulation with GA-treated SKOV3.A2 cells

To determine the immunogenicity of tumor cells treated with GA for 3 and 20 h, 10^5 GA-treated SKOV3.A2 cells were washed and co-cultured with 2×10^6 plastic non-adherent PBMC at a responder to stimulator ratio of 20:1, in RPMI 1640 medium containing 10% HS. Control SKOV3.A2 stimulators were not treated with GA. In the plastic non-adherent cells, $CD13^+$ cells ranged between 1–2%, thus in the culture they were present in significantly smaller numbers than tumor cells. Sixty IU/ml of IL-2 (Cetus) were added in each well 48 h after stimulation. Twenty-four hours later lymphocytes were separated from tumor cells and expanded in complete RPMI medium with 90-IU/ml IL-2. To expand responders all lymphocyte cultures were stimulated 1 week later with plastic bound OKT3 mAb. Proliferation levels of tumor stimulated lymphocytes were determined by counting the recovered viable cells in each culture at defined intervals. The lytic activity of all cultures was determined 3 weeks after tumor stimulation. To establish the specificity of epitope recognition we determined the ability of SKOV3.A2 stimulated T cells to recognize HER-2 peptides, E91, D113, E75, F57, E89, and C85 presented on T2 cells in CTL assays. Specific peptide recognition was determined using as control T2 cells which were not pulsed with peptide (T2-NP). Induction of a CTL response to a peptide was considered specific when the difference between the specific lysis of T2 cells pulsed with a particular peptide and the specific lysis of T2-NP cells plus 2 SD was > 10% as described [22, 28].

Results

Geldanamycin treatment of SKOV3.A2 cells enhanced the rate of HER-2 ubiquitination and decreased the HER-2 levels

To characterize the relationship between HER-2 degradation and ubiquitination, we wanted to identify: (a) specific time points when HER-2 levels, in the presence of GA, decreased significantly (i.e. by more than 50%) and (b) the type of ubiquitination (poly, oligo-), of HER-2 synthesized in the presence of GA for intervals exceeding the protein half-life. We rationalized that characterization of HER-2 protein levels at different time points of its ubiquitination and comparison with the levels of recognition by indicator CTL at the same time points may provide an answer to the question whether HER-2 CTL epitope presentation is dependent on the precursor protein concentration and/or its ubiquitination.

Equal numbers of SKOV3.A2 cells were treated with either 3 μM GA, 3 μM GA + 10 μM LC, or DMSO (in controls) for 3, 10, and 20 h and used to determine the effects of GA-treatment on HER-2 expression and ubiquitination. These time intervals were chosen as representative points for expression of poly-Ub^+-HER-2 (3 h) and poorly- Ub^+-HER-2 (10 and 20 h) from preliminary studies in our laboratory using variable GA concentrations. HER-2 protein (ubiquitinated or not) was immunoprecipitated from cell lysates with *Ab5*. Non-ubiquinated (Ub^-)-HER-2 was identified in Western blotting with *Ab3*. *Ab3* reacts with the C-terminal domain of non-ubiquitinated (Ub^-)-HER-2. Thus, *Ab3* does not detect Ub-HER-2. In contrast, *Ab5* reacts with an epitope on the extracellular domain of HER-2 which is not masked by ubiquitin attachment. *Ab5* reactivity with HER-2 is not affected by intracellular domain modification and precipitates both (Ub^-)-HER-2 and Ub^+-HER-2 [19].

Three hour GA-treatment decreased the level of (Ub^-)-HER-2 precipitated with *Ab5* by 71% compared with GA non-treated control. This was indicated by immunoblot analysis (Fig. 1A, lane 2 vs. 1). Similar (Ub^-)-HER-2 levels were found in the GA and GA + LC treated samples (lane 2 vs. 3). These quantitative relationships are shown in the legends of Figs 1A and 1B and were reproduced in several independent

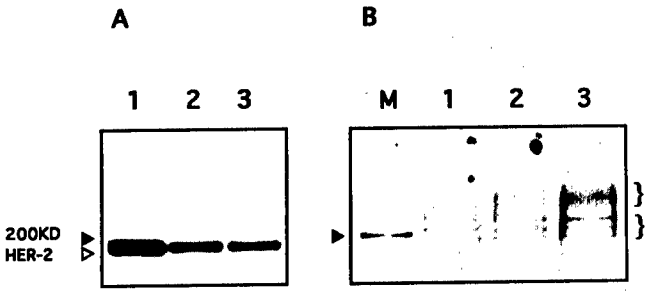


Fig. 1. Geldanamycin enhanced the rate of ubiquitination of HER-2 protein in SKOV3.A2 cells. (A) Immunoblots for HER-2 protein; (B) immunoblots for ubiquitinated protein. SKOV3.A2 cells were lane (1) non-treated, (2) treated with 3 μM GA for 3 h, (3) 3 μM GA + 10 μM LC for 3 h; followed by immunoprecipitation, Western blotting and densitometric analysis of HER-2 and Ub-HER-2 as described in 'Materials and methods'. The densitometric values for the HER-2 bands in Fig. 1A are as follows: (1) 3400, (2) 980, (3) 920. The amount of HER-2 and Ub-HER-2 in each lane corresponds to the amount precipitated from 3.6×10^6 cell equivalents, or 1.0 mg lysate total protein.

experiments (data not shown). The electrophoretic mobility of the HER-2 species recognized by *Ab3* in the analysis shown in Fig. 1A (185 kD) corresponds to (Ub^-)-HER-2.

To determine whether the decrease in (Ub^-)-HER-2 corresponded to an increase in ubiquitinated HER-2 (Ub^+)-HER-2, equal amounts of the same *Ab5* precipitated HER-2 isolated from cell lysates were examined for the presence of the Ub–HER-2 complexes by probing with anti-Ub antibodies. In all samples a triplet of bands were present with M.W. in the range of 200 kD (Fig. 1B, lower brace). These bands were absent from control blots where the anti-Ub antibody was omitted (data not shown). It is possible that they represent oligo-Ub^+-HER-2 since binding of one Ub chain adds 8–9 kD to the mass of protein (i.e. 185 + (3 × 9) = 212 kD). Low levels of poly Ub^+-HER-2 were found in the control and GA-treated samples (Fig. 1B, lanes 1 and 2, upper brace). In GA + LC treated samples the poly Ub^+-HER-2 levels were significantly higher than in GA-treated samples (lane 3 vs. 2) suggesting that poly Ub^+-HER-2 is degraded by proteasome. The results also show that only a part of HER-2 undergoes ubiquitination in the presence of GA (Figs 1A and 1B, lane 2 vs. 3). In 3 h GA-treated cells the major loss of (Ub^-)-HER-2 clearly was not compensated by the slight increase in the Ub^+-HER-2 levels. We infer from this that some HER-2 molecules are degraded faster at 3 h in response to GA compared with control (lane 1 vs. 2). Because of the 'smear-like' [19] appearance of the poly (Ub^+)-HER-2 bands, we could not precisely estimate from densitometric analysis whether differences in (Ub^-)-HER-2 levels in GA + LC treated vs. GA-treated cells (Fig. 1A, lane 3 vs. 2) are accounted for by the level of poly (Ub^+-HER-2 (Fig. 1B, lane 3 vs. 2). Thus, we could not exclude the possibility that in short-term GA-treated SKOV3.A2 cells some HER-2 molecules may be degraded by a pathway different from the PA.

Since stable Ag presence was reported to be critical for CTL recognition [29–31], we determined the long-term effects of GA on the expression of HER-2 protein. SKOV3.A2 cells were treated with GA for 10 and 20 h. HER-2 expression was compared with control cells treated with DMSO for the time interval. To minimize the potential de-ubiquitinating activity in lysates when HER-2 levels were low, isopeptidase inhibitor N-methyl-maleimide (N-mM) was added at the time of tumor lysis. A weakly positive oligo-Ub^+-HER-2 band was detected at 210 kDa in 10 and 20 h GA-treated cells. The results are shown in duplicate (Fig. 2A, lanes 2–6). Poly Ub^+-HER-2 was not detected in 10 and 20 h GA-treated cells (Fig. 2A vs. Fig. 1B). The results also show that the amount of *Ab5* precipitated (Ub^-)-HER-2 at 10 h GA incubation was minimal compared with control (Fig. 2B, lanes 3, 4 vs. 1, 2), suggesting that most of the existent HER-2 was degraded. Interestingly, the levels of (Ub^-)-HER-2 after 20 h GA treatment, although lower than of HER-2 in control cells, were higher than the levels of (Ub^-)-HER-2 at 10 h (Fig. 2B, lanes 5, 6 vs. 3, 4). This suggested that HER-2 protein continued to be synthesized between 10 and 20 h GA treatment. The experiment was repeated and the results were confirmed. These results also indicate that HER-2 in 20 h GA-treated cells is less ubiquitinated than in 10 h GA-treated cells (Fig. 2A, lanes 3, 4 vs. 5, 6).

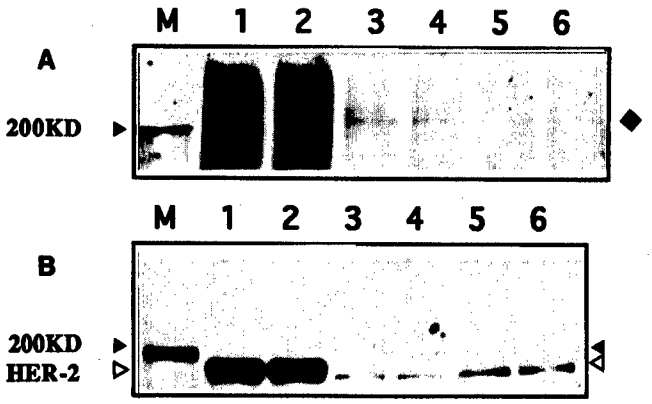


Fig. 2. The HER-2 chain synthesized in the 10 and 20 h presence of GA is poorly ubiquitinated. (A) Immunoblots for ubiquitin; (B) immunoblots for HER-2. SKOV3.A2 cells were cultured to half confluence to ensure that their growth is not inhibited, detached with trypsin, mixed and replated in the presence (lanes 3–6) or absence (lanes 1 and 2) of 3 μM GA. Cells were cultured in the absence of GA for 20 h (lanes 1 and 2), or in the presence of GA for 10 h (lanes 3 and 4) or 20 h (lanes 5 and 6). The experiment was performed in duplicate. The densitometric values for the HER-2 bands 1–6 were 4026, 3932, 84, 86, 231, and 261, respectively. The amount of HER-2 in each lane corresponds to the amount precipitated from 1.6×10^6 cell equivalents or 0.6 mg lysate protein. To facilitate identification of Ub-HER-2 bands, the amount of Ub-HER-2 loaded per lane corresponds to 3.2×10^6 cells equivalents or 1.2 mg protein, i.e. was twice the amount loaded per lane in Fig. 2B. The densitometric values for the Ub-HER-2 bands (marked ♦) were, 620, 720, 230, 260, 128, and 128 respectively. M indicates molecular weight marker, ◀ HER-2 protein in control cells, Δ HER-2 protein in GA-treated cells.

GA-treatment downmodulated HER-2 while increasing HLA-A2 expression

To determine whether GA treatment affected HLA-A2 expression, SKOV3.A2 cells were treated with GA for 3 and 20 h, then stained and analyzed for the expression of HER-2 and HLA-A2. The results are summarized in Table 1. The results show that GA induced a 10-fold decrease in the expression levels of surface HER-2 within 3 h, which was paralleled by a 7-fold increase in the levels of expression of HLA-A2. Further incubation with GA for up to 20 h induced a further 4-fold decrease in the levels of surface expressed HER-2 but did not increase the levels of HLA-A2 compared with 3 h GA treatment. These results suggest that enhanced proteasome mediated protein degradation may lead to an increase in the amount of peptides generated from degraded

Table 1. Effects of GA-treatment on HER-2 and HLA-A2 expression on SKOV3.A2 cells

Time in culture with geldanamycin (h)	Surface HER-2 (fold decrease)	Surface HLA-A2 (fold increase)	HLA-A2 / HER-2
0	3260 (1.0)	149 (1.0)	0.05
3	340 (9.6)	1078 (7.3)	3.17
20	81 (40.3)	780 (5.3)	9.63

SKOV3.A2 cells were treated in culture with 3 μM of GA for the indicated intervals, using DMSO as control. At the indicated times, cells were detached and stained with HER-2 and BB7.2 mAb, followed by goat anti-mouse FITC. The results indicate the anti-log values for the specific mean channel-fluorescence (MCF) for each surface Ag. The specific MCF was calculated by subtracting the control MCF-values for staining with isotype control IgG from the MCF-values obtained at staining with Ab2 and BB7.2.

protein, of which a part may bind HLA-A2 and contribute to its increased expression. SKOV3.A2 cells did not express ICAM-1 but did constitutively express B7.1, although at markedly lower levels than DC. We also determined in parallel that 3 and 20 h GA treatment did not affect tumor cells B7.1 expression and did not induce ICAM-1 expression (data not shown). Thus, in the live recovered cells, GA downregulated most of TM-HER-2, but increased HLA-A2 expression.

Presentation of the HER-2 CTL epitope, E75, requires proteasomal activity

To identify which of the MHC-I processing pathways, proteasome (PA) or cytosolic proteases, contribute to E75 CTL epitope formation, SKOV3.A2 cells were treated with either LLnL (proteasome inhibitor) or E64 (a cysteine-protease inhibitor that is without effect on the proteasome). SKOV3.A2 cells prepared exactly the same way as for HER-2 immunoprecipitation experiments were pre-incubated for 2 h either with 50 μM LLnL, 50 μM LLnL plus 10 μM GA, 50 μM E64 or DMSO as control. The cells were then detached with trypsin. This approach was used in all experiments including when Act-D and CHX were used to eliminate the dead and damaged cells before incubation with CTL. Thus the viability of SKOV3.A2 cells used in all experiments exceeded 90%. Afterwards, the cells were labeled, and incubated with indicator CTL-1. E75-specific CTL-1 line specifically recognized E75 presented on T2 cells. CTL-1 also lysed significantly better SKOV3.A2 cells compared with the SKOV3 cells (Fig. 3A) suggesting that it recognized an epitope presented by HLA-A2. Furthermore, recognition of SKOV3.A2 by CTL-1 was inhibited by T2 pulsed with E75, but not with T2 which were not pulsed with exogenous peptide (T2-NP), or T2 which were pulsed with one irrelevant peptide suggest-

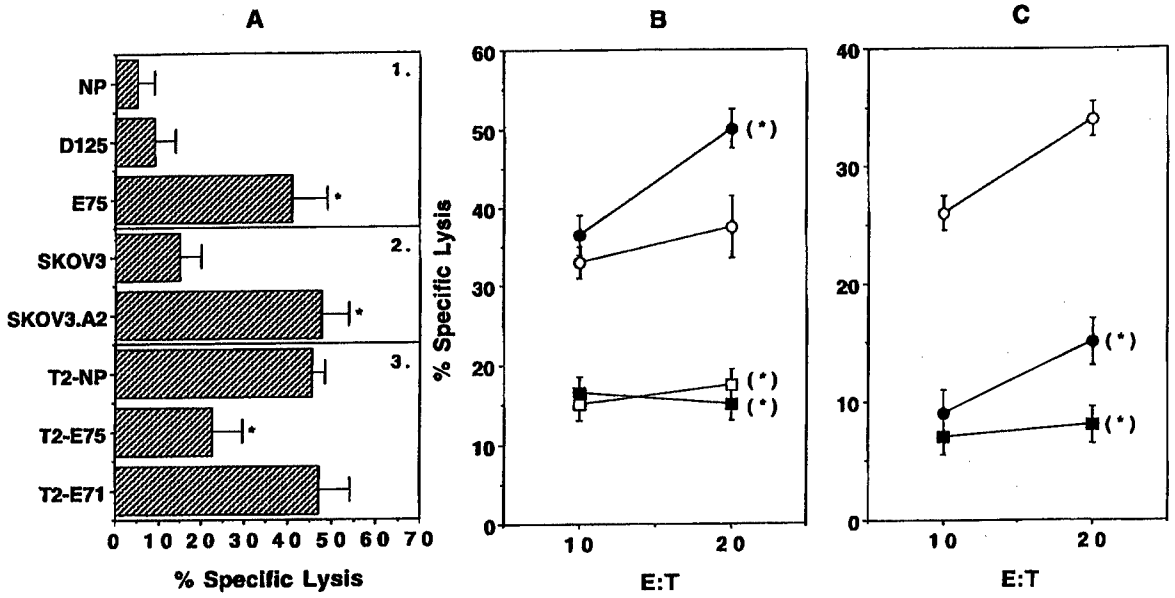


Fig. 3. (A) Specificity of E75 recognition by indicator CTL-1 in direct target lysis and cold-target inhibition assays. (A1) Targets were T2 cells pulsed with 25 μg/ml of D125 or E75, N.P. indicates no peptide; (A2) targets were SKOV3 cells (HLA-A2$^-$) or SKOV3.A2 cells (HLA-A2$^+$). E:T ratio was 25:1; (A3) cold-target inhibition of SKOV3.A2 lysis by CTL-1 by E75 pulsed T2 cells. Cold:hot ratio was 20:1 T2-NP and T2-E71 are negative inhibition controls. *$p < 0.05$ compared with T2-NP. T2 cells were pulsed with either no peptide (NP), or E75, or E71. T2 were pulsed with each peptide at 25 μg/ml for 2 h before being used in the experiment. (B) Recognition of the HER-2, CTL epitope E75 (369–377) is dependent on proteasomal activity and it is potentiated by inhibition of cysteine-proteases. LLnL but not E64 inhibits recognition of SKOV3.A2 cells by CTL-1. Results of 5 h CTL assay, (O) control, no inhibitor, (●) E64, (□) LLnL, (■) GA + LLnL. E:T was 20:1. *$p < 0.05$ compared with control (O). (C) SKOV3.A2 pretreatment with Act-D alone or together with CHX inhibits its recognition by CTL-2 (O) control untreated, (●) Act-D, (■) Act-D + CHX. E:T = 20:1. *$p < 0.05$ compared with control (O). (A–C) results of separate experiments. Experimental details are presented in 'Materials and methods'.

ing that CTL-1 recognized either endogenously generated E75, or a structurally similar epitope. Similar results were observed with CTL-2 obtained from the other donor (data not shown).

The results (Fig. 3B) showed that LLnL markedly inhibited CTL-1 recognition whereas E64 did not. This suggested that the proteasome is involved in CTL-1 recognized epitope formation. The levels of CTL-1 recognition of SKOV3.A2 were lower in control than in E64 treated cells. This suggested that E75 is not formed by cysteine protease degradation of HER-2 but rather cysteine proteases inhibited E75 formation. These results indicated that in SKOV3.A2 cells, the proteasomal pathway was functional and participated in E75 formation.

To address whether E75 recognition is dependent on the synthesis of novel precursor, processing, and presentation elements, SKOV3.A2 cells were treated in culture for 7 h (i.e. the HER-2 protein half-life) with 5 μg/ml Act-D alone, or in the presence of 100 μg/ml cycloheximide (CHX). Afterwards, cells were washed, detached with trypsin, labelled, and incubated with CTL-1 in a 5 h CTL assay without addition of Act-D and CHX. The results (Fig. 3C) show that Act-D dramatically inhibited E75 recognition compared with control, DMSO only treated cells, tested in parallel. CHX enhanced the inhibitory effect of Act-D suggesting that E75 recognition from SKOV3.A2 cell is also dependent on protein synthesis. Thus, the Act-D effects suggest a requirement for mRNA and protein synthesis by the tumor for CTL epitope recognition.

GA treatment enhanced E75 recognition on SKOV3.A2 cells

GA-induced acceleration of HER-2 degradation raised the question whether faster HER-2 degradation leads to increased E75 presentation. To address this question we analyzed recognition of GA-treated SKOV3.A2 cells for 3 and 20 h in parallel with control untreated cells by CTL-1. Results in Fig. 4A show that 3 h GA treatment did not increase SKOV3.A2 lysis compared with controls. These results also indicated that the pre-existent HER-2, whose levels dramatically decreased during the first 3 h of GA treatment, (by 70%, Fig. 1A) did not lead to a substantial increase in E75 presentation. In contrast, lysis of SKOV3.A2 markedly increased by > 100% compared with controls when GA treatment was continued by 20 h (Fig. 4A). As indicated before, SKOV3.A2 were first grown to half-confluency, then treated with GA in culture, then detached with trypsin and then labeled and used as targets. Thus the differences in lysability did not reflect differences in viability of the targets. These experiments were repeated several times with various CTL lines obtained ei-

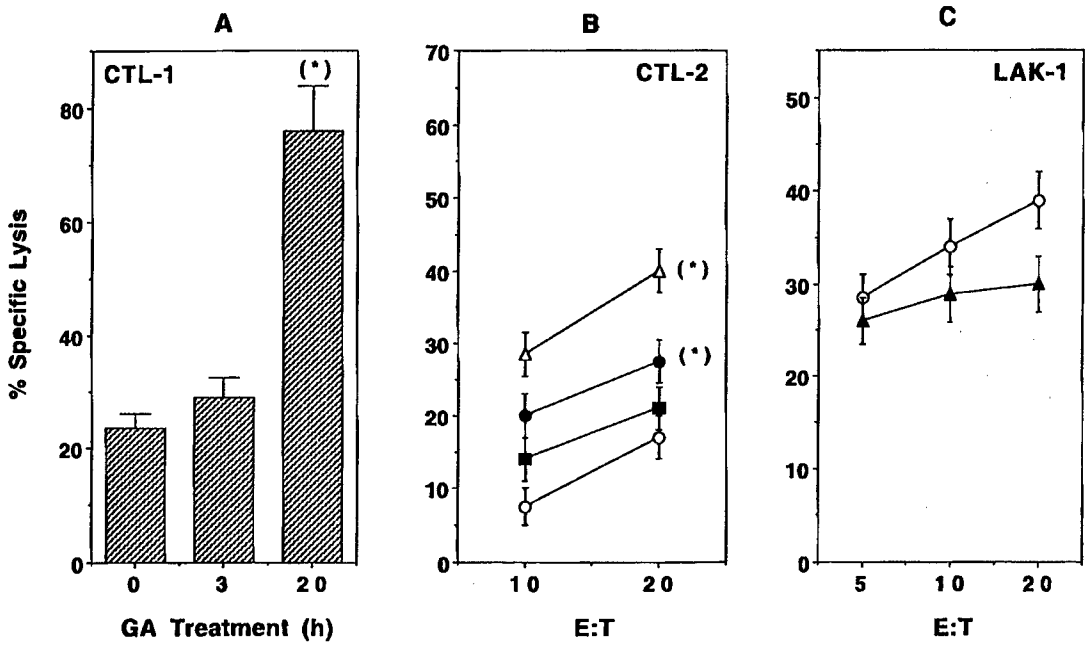


Fig. 4. (A) Increased presentation of E75 by 20 h GA-treated SKOV3.A2 cells. SKOV3.A2 cells were pre-treated with GA for 0, 3, and 20 h then washed and incubated with CTL-1 for 5 h. Results are expressed as % specific lysis. E:T ratio was 10:1. (B) SKOV3.A2 cells were treated with GA for 0, (O), 3 (▫), 8 (●) and 20 h (□). CTL-2 were used as effectors at 10:1 and 20:1. *indicates significant increase in target lysis compared with control (O) non GA-treated targets. (C) GA treatment does not enhance the lysability of SKOV3.A2 cells by LAK cells. (O) control untreated SKOV3.A2, (▲) 20 h GA-treated SKOV3.A2. SKOV3.A2 cells were incubated with LAK cells from donor 1 generated by culture of PBMC in 1000 U/ml IL-2 for 2 weeks. All results are from 5 h CTL assays performed in triplicate and show specific lysis ± S.D.

ther from tumor associated lymphocytes or from PBMC with similar results (not shown) regarding the effects of 3 h GA treatment.

To better clarify the question of the timing of increased HER-2 epitope presentation, SKOV3.A2 cells were treated with GA for 0, 3, 8 and 20 h and tested for recognition by CTL-2 at two E:T ratios. The results in Fig. 4B show that similarly with CTL-1, 3 h GA-treatment did not enhance SKOV3.A2 sensitivity to CTL-2 at two E:T ratios. However, the 8 h GA-treatment significantly enhanced the lysis of SKOV3.A2 cells by CTL-2 by 30–40% at both E:T ratios compared with control, DMSO treated cells. Again 20 h GA-treatment of SKOV3.A2 cells lead to an at least 2-fold increase in lysis by CTL-2 compared with control targets confirming the results with CTL-1.

These results suggest a delay in the increased presentation of E75 induced by GA, and the possibility that the polyubiquitinated HER-2 is not the source of this epitope. To eliminate the possibility that higher lysis represented increased lysability of the tumor due to GA treatment, we tested, in a parallel experiment, tumor lysis by activated LAK cells. LAK cells were generated from PBMC of the same donor 1 (LAK-1) thus they were autologous with CTL-1. LAK-1 lysis was similar for both the control untreated and 20 h GA-treated SKOV3.A2 cells at two E:T ratios and was slightly higher for the control targets than for GA-treated cells at the highest ratio (Fig. 4C). This indicated that increased lysis by CTL-1 does not reflect higher sensitivity of GA-treated targets to non-specific effectors. The higher sensitivity of 20 h GA-treated SKOV3.A2 compared with control SKOV3.A2 to CTL-1 than to LAK-1 confirmed that sensitivity to CTL did not reflect differences in lysability of the targets. Thus increased E75 presentation from SKOV3.A2 cells was a late event subsequent to GA treatment and involved the HER-2 chain synthesized in the presence of GA but not the existent chain rapidly ubiquitinated by GA.

Enhanced immunogenicity for CTL induction by 20 h GA-treated SKOV3.2A cells

To address the effects of GA-accelerated degradation of HER-2 in CTL induction, we used SKOV3.A2 cells as stimulators and healthy donors PBMC as responders. To minimize the contribution of professional APC, responders consisted of the plastic non-adherent fraction of PBMC. The SKOV3.A2 stimulators were not irradiated to allow their proliferation, HER-2 synthesis, and peptide presentation.

To determine how stimulation with GA-treated tumor affected induction of cytolytic function, responders were assessed for their ability to recognize six HER-2 peptides in the same assay. One of these peptides was from the N-terminal end (E91), three of these peptides were from the extracellular domain (D113, E75 and F57) and two of these peptides were from the intracellular domain (E89, C85). CTL from each donor were tested for recognition of the dominant CTL epitope E75 and of the subdominant epitopes C85 and F57 [32, 33] in the same experiment. F57 was identified in an independent study to be recognized by ovarian CTL-TIL [33]. HER-2 peptides D113, E91, and E89 were used as controls. D113 binds to HLA-A2 with higher affinity than E75. D113 was found in our studies and other previous studies to induce peptide specific CTL [28, 34]. Similarly E89 was found occasionally to be recognized by tumor-reactive CTL [22]. Cytotoxicity values for each peptide were considered positive when they were at least 10 percentage points greater than mean values for specific lysis in the absence of peptide. All three CTL cultures were tested in the same experiment. These cultures were ~ 80% $CD8^+$ (data not shown).

Of six HER-2 peptides tested, only E75 was recognized by donor 1 CTL induced with 20 h GA-treated SKOV3.A2 (20 h-GA-CTL) (Figs 5A and 5B). Donor 1 CTL induced by stimulation with 3 h GA-treated SKOV3.A2 (3 h-GA-CTL), specifically recognized D113 and borderline E91 and E75 (Fig. 5A). None of these peptides were specifically recognized by CTL from donor 1 stimulated with control untreated CTL (0 h-GA-CTL).

A similar pattern of recognition was observed with donor 2. To minimize the contribution of transmembrane HER-2 CTL were induced by stimulation with 15 h GA-treated SKOV3.A2. Fifteen hour GA-CTL-2 showed specific recognition only of E75 and G89 in a 5 h CTL assay while recognition of E91 and C85 was borderline and considered non-specific (Fig. 6A). Thus similarly with donor 1, E75-specificity was induced in donor 2, by stimulation with 15 h GA-treated tumor.

To address the question whether HER-2 peptide specific CTL were present in 0 h-GA-CTL and 3 h-GA-CTL, but their numbers were too small or their lytic activity was too weak to be detected in 5 h assays, 0 h-GA and 3 h-GA, donor 2 CTL were tested against peptide pulsed T2 in a long (20 h) CTL assay. The results in Fig. 6B show that 0 h-GA CTL specifically recognized only C85. In contrast, 3 h GA-CTL recognized specifically D113, E75, F57 and C85. Compared with 0 h-GA-CTL, there was an increase in recognition of C85 in 3 h-GA-CTL as well as in the recognition of D113. This confirmed that GA-treatment enhanced the immunogenicity of tumor cells for peptide specific CTL.

Induction of HER-2 peptide specificities by 20 h GA-treated tumor suggested that HER-2 synthesized in the presence of Ga, is more immunogenic for $CD8^+$ cell activation than preexistent HER-2. It is interesting to note that the HLA-A2 levels were higher in 3 and 20 h GA-treated cells than in the tumor cells not treated with GA although the levels of HER-2 were lower. This is suggested by the high linear correlation between % lysis and the HLA-A2/HER-2 ratio in donor

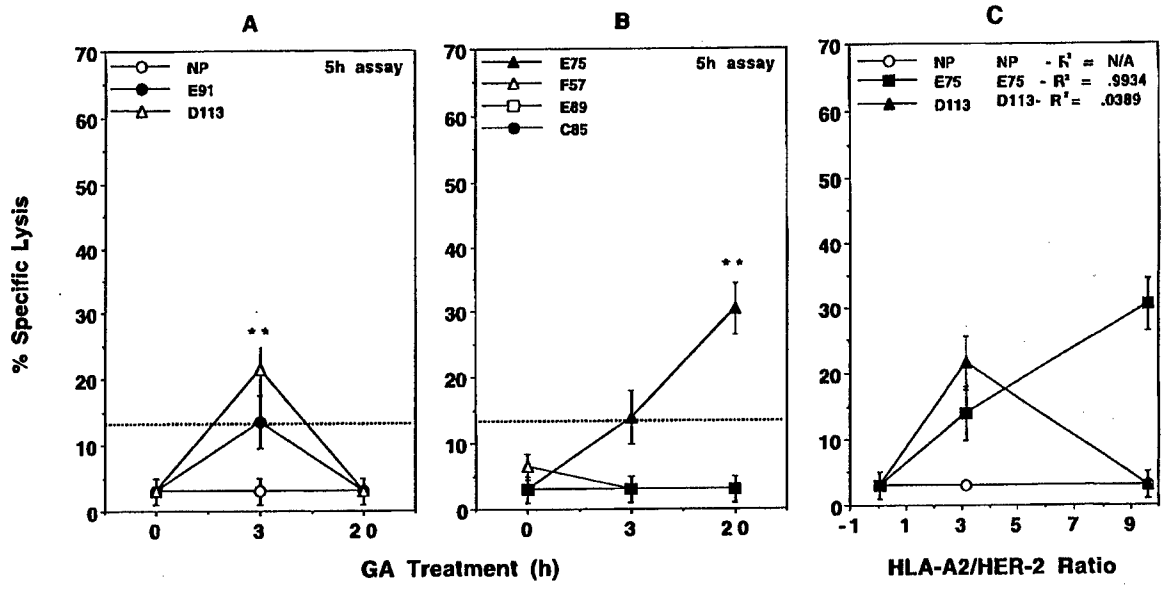


Fig. 5. (A, B) Recognition of HER-2 peptides E91, D123, E75, F57, E89 and C85 by CTL from donors 1 and 2 induced by stimulation with control (0 h), and GA-treated SKOV3.A2 cells for 3 h, and 20 h respectively. CTL induction, propagation and specificity determinations were performed as described in 'Materials and methods'. Recognition of all HER-2 peptides by CTL from each donor was determined in the same experiment at an 20:1, E:T ratio. T2 cells were pulsed with 25 μg/ml of each peptide. T2 cells which were not pulsed with peptide (NP) were used as control. % (specific lysis) was calculated as described in 'Materials and methods'. Results are shown as % specific lysis ± S.D. The horizontal dotted line indicates the cut-off levels for definition of a positive response. **$p < 0.05$ compared with T2-NP + SD). (C) Results are shown as % (specific lysis) ± S.D. vs. the ratio of HLA-A2/HER-2 expression for the 0 h control and the 10 and 20 h GA-treated SKOV3.A2 cells. Linear regression analysis showed a higher linear correlation ($R^2 = 0.9934$) between the % lysis for T2-E75 and the HLA-A2/HER-2 expression for cells exposed to GA for various lengths of time.

1 (Fig. 5C) and the similarly high correlation for E75 and E89 in donor 2 (Fig. 6, legend). The increased HLA-A2 expression, probably together with selective changes in epitope expression may allow the tumor to reach the threshold for CTL activation. Thus although additional, as yet unknown GA-induced or activated factors and processes may contribute to

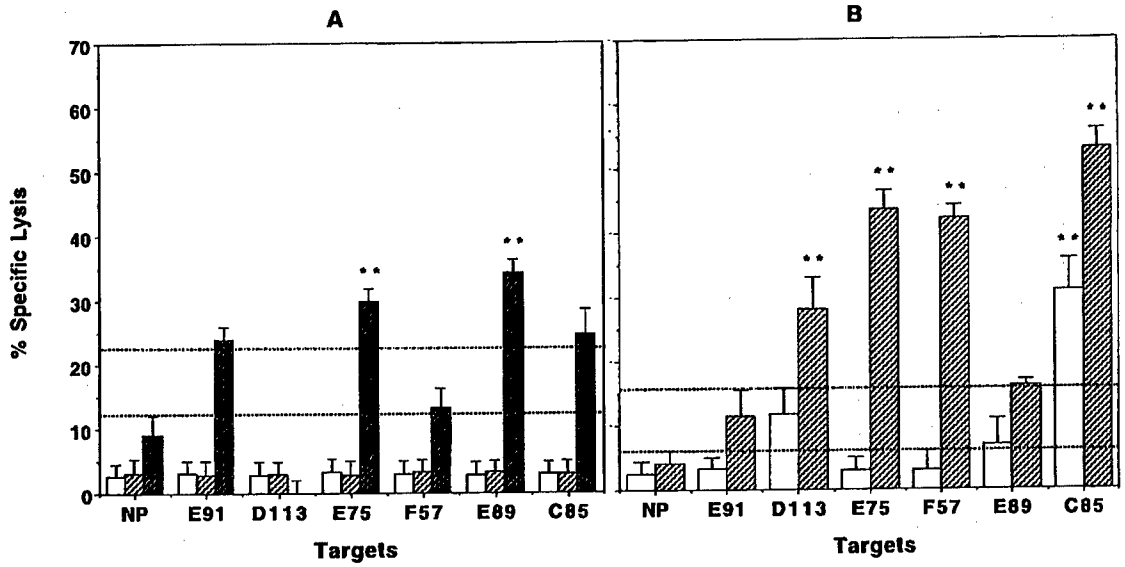


Fig. 6. Recognition of HER-2 peptides by CTL from donor 2 induced by stimulation with SKOV3.A2 cells treated with GA 0 h (□), 3 h (▨), or 15 h (■). (A) 5 h CTL assay; regression analysis showed a high correlation coefficient ($R^2 = 0.891$ in exponential regression) between E75 and G89 recognition and the HLA-A2/HER-2 ratios, suggesting preferential expression of such epitopes in GA-exposed cells. (B) 20 h CTL assay. E:T was 20:1. The upper horizontal dotted lines indicate the 10% cut-off level above lysis of T2-NP targets ± S.D. Results indicate mean ± S.D. of a CTL assay performed in triplicate. **%specific lysis – SD) indicate significant recognition of a HER-2 peptide compared with targets which were not pulsed with peptide + SD ($p < 0.05$).

SKOV3.A2 immunogenicity, these results show that increased epitope presentation, subsequent to faster precursor self-protein degradation, when precursor synthesis is not inhibited, may enhance the immunogenicity of tumor cells.

Discussion

We investigated the consequences of increased rate of degradation of a self-protein, HER-2, expressed in tumor cells on CTL recognition. Our results provide a first insight into the relationship between expression of HER-2 protein, CTL recognition, and tumor antigenicity. The strategy to increase the rate of HER-2 degradation was to destabilize HER-2:grp94 by treating tumor cells with GA. The SKOV3.A2 cells were always grown to half-confluence. This assured that the protein synthesis was not inhibited because of growth arrest. By allowing protein and HER-2 synthesis to continue in both control and GA-treated cells, epitope presentation was not limited by the availability of the precursor protein. We found that E75 presentation increased only in 20 h GA-treated cells although the levels of HER-2 protein expression were lower than in control. These results show that HER-2 synthesized in the presence of GA can be an important precursor for CTL epitope formation.

E75-specific CTL showed higher lysis of 20 h GA-treated SKOV3.A2 cells than of 3 h GA-treated cells. Since in the former cells the levels of (Ub^+)-HER-2 were significantly lower than in the latter, these results suggest that E75 was presented more efficiently from the 20 h GA-treated cells than from 3 h GA-treated cells. In 20 h GA-treated cells, HER-2 was oligoubiquitinated while in 3 h GA-treated cells it was polyubiquitinated. The HER-2 chain in 20 h GA-treated cells was less ubiquitinated compared even with 10 h GA-treated cells. One possibility to explain the weak enhancement of E75 presentation from the polyubiquitinated HER-2 is that the rapid HER-2 polyubiquitination leads to excess epitope, which may overcome the capacity of the transport system as suggested by Goth *et al.* [35]. The excess precursor may be then degraded by other proteases as suggested by enhanced E75 recognition in the presence of cysteine protease inhibitor E64. Thus, a lag period may be required for synthesis of additional transport elements. In fact we found that when CTL were added to the tumor 15–30 min after GA treatment, tumor lysis decreased compared with control targets (Swearingen, preliminary data).

A second possibility is that the kinetics of epitope presentation from oligo Ub-HER-2 may be faster than from polyUb-HER-2. This possibility deserves further investigations. Rapid epitope presentation was previously reported for Ub fusion proteins (oligo-Ub) imported in APC [35–37]. However, there was an important difference between HER-2 and the Ub fusion proteins. HER-2 was C-terminally ubiquitinated while the Ub-fusion proteins were N-terminally polyubiquitinated. Since E75 is located in the extracellular domain, our results suggest that C-terminal ubiquitination impacts on presentation of N-terminal epitopes. The enhanced recognition by SKOV3.A2-induced CTL of E75, and E89 in 5 h assays, and of D113, E75, F57 vs. C85 in 20 h assays, suggested that both extracellular (ECD) and intracellular (ICD) domains of HER-2 are targeted to the MHC-I processing pathways.

An important finding of these studies was that tumor cells expressing higher levels of E75 were more immunogenic than control tumor cells for induction of CTL reacting with HER-2 peptides. This finding suggested a novel pathway for enhancement of tumor immunogenicity. Since in all stimulations, the number of tumor cells was 10^5 while the number of monocytes APC was $\sim 2 \times 10^4$, it is possible that the observed immunogenicity was a direct property of the SKOV3.A2 cells. Induction of specific CTL for E75 by 20 h GA-treated tumors in both donors, suggested that the observed SKOV3.A2 immunogenicity was not the result of non-specific (bystander) or cross-reactive activation of pre-existing effectors as a consequence of allo-recognition. Control stimulators expressed lower levels of HLA-A2 than the GA-treated stimulators. We used as responders two healthy donors which shared HLA-A2 but differed in the additional HLA-A, B, C, molecules expressed. Thus the allostimulatory ability of SKOV3.A2 for each of these donors was different. However, induction of CTL activity for E75 was similar in both donors. If the observed CTL activity was only a bystander effect, then the specificity of CTL from the same donor stimulated with each of the control and GA-treated tumors would have been the same, i.e. donors 1 and 2 would have shown similar recognition specificity.

Three hour GA-treatment increased the levels of HLA-A2 expression on SKOV3.A2 by approximately one order of magnitude (9-fold) compared with control. Continued GA-treatment for 20 h did not increase further the levels of HLA-A2. Although the levels of HLA-A2 were lower than on 3 h GA-treatment, they were still higher than the levels on control cells. Both antigenicity and immunogenicity of SKOV3.A2 with respect to E75 correlated with the increase in the HLA-A2 to HER-2 ratio. However, this correlation suggested that a threshold value is required for CTL activation which may reflect not only quantitative, but also quantitative differences in epitope expression.

Interestingly, both 0 h GA-CTL and 3 h GA-CTL showed some peptide specificity, but only in the long (20 h) CTL assay. This indicated that some effectors are present in the CTL induced by short GA treatment. The questions as to whether this activity reflects a low CTL frequency, incomplete maturation, or the use of alternative effector pathways for killing by such CTL (e.g. TNF-TNFR) should be addressed in future studies.

Our results implicate the HER-2 chain synthesized in the presence of GA in increased E75 presentation. The fact that pretreatment with Act-D, alone or together with CHX, inhibit CTL recognition provided indirect support to this hypothesis. Since GA does not increase HER-2 synthesis, a possibility for the increased E75 presentation is that particular molecular characteristics of the newly synthesized chain increased the yields of E75 formation. The HER-2 protein synthesized in the 20 h presence of GA differed from the protein of control cells by its faster electrophoretic mobility. The reasons for the faster HER-2 mobility are unclear. A recent report indicated that in SKBR3.A2 cells the HER-2 chain synthesized in the presence of GA is poorly glycosylated, is localized in the pre-Golgi compartment, and may not be able to translocate to the cell surface [20]. We found only one HER-2 band in both 10 and 20 h GA-treated cells and higher expression of HER-2 chain on the surface of 20 h GA-treated SKOV3.A2 cells compared with 10 h GA-treated cells. This suggested that the newly synthesized HER-2 is transported to the cell surface, thus HER-2 glycosylation may not be a factor in E75 presentation. In fact, the glucosidase inhibitor castanospermin was without effect on CTL recognition while in the same experiment, the protease inhibitor TPCK inhibited recognition (Swearingen *et al.*, preliminary data). Thus, glycosylation may not be a factor in HER-2-epitope presentation.

One hypothesis for the differences in electrophoretic mobility is that the faster HER-2 mobility of HER-2 may be due to the fact that the chain synthesized in the presence of GA is not phosphorylated at the active serine/threonine sits as it is in control SKOV3.A2 cells [23]. SER/Thr phosphorylated proteins are characterized by slower electrophoretic mobility compared with their non-phosphorylated counterparts (O'Brian, personal communication). The possibility that in GA-treated cells HER-2 is not phosphorylated, as in the control cells, is currently under investigation. Recent studies show that HER-2 in SKOV3.A2 cells is not constitutively phosphorylated at Y1248 but is phosphorylated at Thr686 [23, 28]. It is not clear whether Y1248 and/or Thr686 phosphorylated HER-2 are good substrates for epitope formation. Y1248 HER-2 heterodimerize with HER-1 (EGF-R) and/or HER-3 leading to endocytosis and its degradation in the lysosomal compartment. Lysosomal degradation is known to generate peptides for MHC-II presentation. The use of antibodies or of tyrosine kinase agonists, such as EGF, in high concentrations to accelerate downmodulation of HER-2 may not be likely to result in HER-2 gaining access to the endogenous MHC-I presentation pathway of the tumor to activate CTL.

The ability of pharmacologic mediators of dephosphorylation such as GA to enhance tumor sensitivity to CTL and potentiate its immunogenicity may have implications in identification of molecular mechanisms of tumor immunogenicity *in vivo*. Ongoing studies are characterizing the nature of the HER-2 epitope precursor, which leads to immunogenic presentation of E75 and of other HER-2 epitopes. Preliminary results in our laboratory indicate that changes in the tyrosine and serine/threonine phosphorylation of HER-2 affect CTL epitope presentation and tumor immunogenicity (Castilleja *et al.*, manuscript in preparation). Since HER-2 is subjected to phosphorylation at different sites under the influence of various agonists during tumor progression, it will be important to determine how environment induced HER-2 post-translational modifications control tumor antigenicity and immunogenicity.

Acknowledgements

The authors wish to thank Dr. Len Neckers, National Institute of Health, for technical advice on the ubiquitination analysis studies, and Dr. Thomas Braciale (University of Virginia) for fruitful discussions. This research was supported in part by Grants DAMD 4313/7098. Peptide synthesis was supported in part by the NIH core grant CA-16672.

Note added in proof

When this paper was submitted for publication Ulrich, S. *et al.* (Nature, 404(6779), 770–774 13/14/2000 issue) reported that metabolically unstable and ubiquitinated proteins are major sources of CTL epitopes. This report concurs with the findings of Drs. Yewdell and Bennink's group, by extending the applicability of DRIP concept to self-proteins, which are tumor antigens.

References

1. Speiser DE, Miranda R, Zakarian A, Bachmann MF, McKall-Faienza K, Odermatt B, Hanahan D, Zinkernagel RM, Ohashi PS: Self-antigens expressed by solid tumor do not efficiently stimulate naive or activated T cells: Implications for immunotherapy. J Exp Med 186: 645–653, 1997
2. Onrust SV, Hartl PM, Rosen SD, Hanahan D: Modulation of L-selectin ligand expression during an immune response accompanying tumorigenesis in transgenic mice. J Clin Invest 97: 54–64, 1996
3. O'Connell J, Sullivan GC, Collins JK, Shanahan F: The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 184: 1075–1082, 1996
4. Seung LP, Rowley DA, Dubey P, Schreiber H: Synergy between T cell immunity and inhibition of paracrine stimulation causes tumor rejection. Proc Natl Acad Sci USA 92: 6254–6258, 1995

5. Kono K, Halapi E, Hising C, Petersson M, Gerdin E, Vanky F, Kiessling R: Mechanisms of escape from CD8+ T cell clones specific for the HER-2/neu proto-oncogene expressed in ovarian carcinomas: Related and unrelated to decreased MHC class 1 expression. Int J Cancer 70: 112–119, 1997
6. Maeurer MJ, Gollin SM, Martin D, Swaney W, Bryant J, Castelli C, Robbins P, Parmiani G, Storkus WJ, Lotze MT: Tumor escape from immune recognition: Lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen. J Clin Invest 98: 1633–1641, 1996
7. Wick M, Dubey P, Doeppen H, Siegel CT, Fields PE, Chen L, Bluestone JA, Schreiber H: Antigenic cancer cells grow progressively in immune hosts without evidence for T cell exhaustion or systemic energy. J Exp Med 186: 229–238, 1997
8. Michalek M, Grant EP, Gramm C, Goldberg KL, Rock KL: A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. Nature 363: 552, 1993
9. Cox JH, Galardy P, Bennink JR, Yewdell JW: Presentation of endogenous and exogenous antigens is not affected by inactivation of e1 ubiquitin-activating enzyme in temperature-sensitive cell lines. J Immunol 154: 511–519, 1995
10. Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg KL: Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I. Cell 78: 761–771, 1994
11. Yewdell JW, Anton LC, Bennink JR: Defective ribosomal products (DRiPs): A major source of antigenic peptides for MHC class I molecules? J Immunol 157: 1823–1826, 1996
12. Pickaut CB: Targeting of substrates to the 26S proteasome. FASEB J 11: 1055–1066, 1997
13. Hicke L: Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. FASEB J 11: 1215–1226, 1997
14. Vinitsky A, Anton LC, Snyder HL, Orlowski M, Bennink JR, Yewdell JW: Generation of MHC class I-associated peptides is only partially inhibited by proteasome inhibitors. Involvement of nonproteasomal cytosolic proteases in antigen processing. J Immunol 159: 554–564, 1997
15. Lopez D, Del Val M: Selective involvement of proteasome and cysteine proteases in MHC class I antigen presentation. J Immunol 159: 5769–5772, 1997
16. Craiu A, Akopian T, Goldberg A, Rock KL: Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. Proc Natl Acad Sci USA 94: 10850–10855, 1997
17. Kupio T, Kankaanranta A, Jalava P, Kronqvist K, Kotkansala T, Weber E, Collan Y: Cystein proteinase inhibitor cystatin A in breast cancer. Cancer Res 58: 432–436, 1998
18. Pasleau F, Grooteclaes M, Gol-Winkler R: Expression of the c-erbB2 gene in the BT474 human mammary tumor cell line: Measurement of c-erbB2 mRNA half-life. Oncogene 8: 849–854, 1993
19. Mimnaugh EG, Chavany C, Neckers L: Polyubiquitination and proteasomal degradation of the p185 receptor protein-tyrosine kinase induced by geldanamycin. J Biol Chem 271: 22796–22801, 1996
20. Chavany C, Mimnaugh E, Miller P, Bitton R, Nguyen P, Trepel J, Whitesell L, Schnur R, Moyer JD, Neckers L: p185erbB2 binds to grp94 *in vivo*. J Biol Chem 271: 4974–4977, 1996
21. Hartmann F, Horak EM, Cho C, Lupu R, Bolen JB, Stetler-Stevenson A, Pfreundschuh M, Waldmann TA, Horak ID: Effects of the tyrosine-kinase inhibitor geldanamycin on ligand-induced HER-2/neu activation, receptor expression and proliferation of HER-2-positive malignant cell lines. Intl J Cancer 70: 221–229, 1997
22. Fisk B, Blevins TL, Wharton JT, Ioannides GC: Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. J Exp Med 181: 2109–2117, 1995
23. Quyang X, Gulliford, Zhang H, Huang GC, Epstein R: Human cancer cells exhibit PKC-dependent c-erb-2 transmodulation that correlates with phosphatase sensitivity and kinase activity. J Biol Chem 271: 21786–21792, 1996
24. Ward NE, Gravitt KR, O'Brien CA: Irreversible inactivation of protein kinase C by a peptide-substrate analog. J Biol Chem 270: 8056–8060, 1995
25. Schuler G, Steinman RM: Dendritic cells as adjuvants for immune-mediated resistance to tumors. J Exp Med 186: 1183–1187, 1997
26. Anderson BW, Peoples GE, Murray JL, Gershenson DM, Ioannides CG: Peptide priming of cytolytic activity to HER-2 epitope (369–377) in healthy individuals. Clin Cancer Res (in press)
27. Yang B, Hahn YS, Hahn CS, Braciale TJ: The requirement for proteasome activity in class I MHC antigen presentation is dictated by the length of preprocessed antigen. J Exp Med 183: 1545–1552, 1996
28. Fisk B, Chesak B, Wharton JT, Ioannides CG: Sequence motifs of human HER-2 proto-oncogene important for peptide binding to HLA-A2. Int J Oncol 5: 51–63, 1994
29. Kundig TM, Shahinian A, Kawai K, Mittrucker HW, Sebzda E, Bachmann MF, Mak TW, Ohashi PS: Duration of TCR stimulation determines costimulatory requirement of T cells. Immunity 5: 41–52, 1996
30. Kundig TM, Backmann MF, Oehen S, Hoffman UW, Simard TJ, Kalberer CP, Pircher H, Ohashi PS, Hengartner H, Zikernagel RM: On the role of antigen in maintaining cytotoxic T cell memory. Proc Natl Acad Sci USA 93: 9716–9723, 1996
31. Cai Z, Sprent J: Influence of antigen dose and costimulation on the primary response of CD8+ T cells *in vitro*. J Exp Med 183: 2247–2257, 1996
32. Ioannides CG, Fisk B, Fan D, Biddison WA, Wharton JT, O'Brien CA: Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER2/neu proto-oncogene. Cell Immunol 150: 225–234, 1993
33. Rongcun Y, Salazar-Onfray F, Charo J, Malmberg KJ, Evrin K, Maes H, Kono K, Hising C, Petersson M, Larsson O, Lan L, Appella E, Sette A, Celis E, Kiessling R: Identification of new HER-2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. J Immunol 163: 1037–1044, 1999
34. Disis ML, Smith JW, Murphy RE, Chen W, Cheever MA: *In vitro* generation of human cytolytic T-cells specific for peptides derived from the HER-2/neu protooncogene protein. Cancer Res 54: 1071–1076, 1994
35. Goth S, Nguyen V, Shastri N: Generation of naturally processed peptide/MHC class I complexes is independent of the stability of endogenously synthesized precursors. J Immunol 157: 1894–1904, 1996
36. Grant EP, Michalek MT, Goldberg AL, Rock KL: Rate of antigen degradation by the ubiquitin-proteasome pathway influences MHC class I presentation. J Immunol 155: 3750–3758, 1995
37. Tobery W, Siliciano RF: Targeting of HIV-1 antigens for rapid intracellular degradation enhances cytotoxic T lymphocyte (CTL) recognition and the induction of *de novo* CTL responses *in vivo* after immunization. J Exp Med 185: 909–920, 1997
38. Peles E, Ben-Levy R, Tzahar E, Liu N, Wen D, Yarden Y: Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. EMBO J 12: 961–971, 1993
39. Chen W, Khilko S, Fecondo J, Margulies DH, McCluskey J: Determinant selection of MHC class I-restricted antigenic peptides is explained by class I-peptide affinity and is strongly influenced by nondominant anchor residues. J Exp Med 180: 1471–1483, 1996
40. Alexander-Miller M, Leggatt GR, Sarin A, Berzofsky JA: Role of antigen, CD8, and cytotoxic T lymphocyte (CTL0 avidity in high dose antigen induction of apoptosis of effector CTL. J Exp Med 184: 485–492, 1996

41. Houghton A: Cancer antigens: Immune recognition of self and altered self. J Exp Med 180: 35–42, 1994
42. Lenschow DJ, Walunas TL, Bluestone JA: CD28/B7 system of cell costimulation. Annu Rev Immunol 14: 233–258, 1996
43. Suzuki I, Fink P: Maximal proliferation of cytotoxic T lymphocytes requires reverse signaling through fas ligand. J Exp Med 187: 123–128, 1998
44. Valitutti S, Muller S, Dessing M, Lanzavecchia A: Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. J Exp Med 183: 1917–1921, 1996

ANTICANCER RESEARCH 22: 1481-1490 (2002)

Helper Peptide G89 (HER-2:777-789) and G89-Activated Cells Regulate the Survival of Effectors Induced by the CTL Epitope E75 (HER-2, 369-377). Correlation with the IFN-γ: IL-10 Balance

TOM V. LEE[1], DENNIS A. JOHNSTON[2], NICHOLAS THOMAKOS[1], TOSHIE HONDA[1], CLAY L. EFFERSON[1] and CONSTANTIN G. IOANNIDES[1,3]

Departments of [1]Gynecologic Oncology, [2]Biomathematics, and [3]Immunology, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030, U.S.A.

Abstract. *The CTL response to Ag expands after priming and subsequently contracts reducing the number of effectors. $CD4^+$ cells are described as regulators of CTL immunity. To elucidate whether $CD4^+$ cells are involved in survival of effector CTL and the survival signals, we used CTL and Th peptides form the HER-2 protooncogene recognized in association with HL-A2 and HLA-DR4, respectively. We analyzed the effect of cells stimulated with G89 (777-789) in survival and expression of lytic function of CTL specific for the epitope E75 (369-377). G89 primed cells (G89-PR) and G89 enhanced expansion and Ag-specific cytolyis of CTL at priming with E75, but inhibited survival of E75-specific CTL at restimulation. These effects were not simply a reflection of the increases in IFN-γ and IL-10, but the ratio IFN-γ/IL-10 modified by G89 differentially regulated the survival of stimulated cells. This suggests that the use of helper antigens in cancer vaccines should be evaluated in the context of their CTL survival inducing effect.*

Induction of cytotoxic T lymphocytes (CTL) responses is dependent upon the presence of MHC-I restricted Ag, corresponding to the CTL epitope. In addition to the CTL epitope, help from CD4+ T cells or cytokines produced by CD4+ cells, was described, in various systems, to be needed for optimization of effector responses by CD8+ cells (1-6). The helper role of the CD4+ cells for induction of CTL responses is still poorly understood. It was associated with : (1) CD40 triggering on macrophages (M0), and dendritic cells (DC) by CD4+ T cells (reviewed in 7); (2) expression of Type 1 and 2 cytokines which compensate for the insufficient cytokine responses by weak CD8+ specific antigens (8) and (3) recruitment of other effectors in addition to CD8+ cells (9).

The effector CTL responses (cytokines and cytolytic function) are frequently quantitated to document vaccine efficacy. The CTL response to Ag expands after priming, because of the increase in the number of Ag-specific CTL, and subsequently contracts to bring down the numbers of effectors to the initial level (reviewed in 10). While this mechanism may be suitable as a protection mechanism for acute viral diseases, which are resolved fast, this is not the case for chronic diseases, such as AIDS or cancer, where the source of infection and the tumor may not be eradicated entirely by the clonal burst of Ag-specific CTL. Thus, increasing the survival of CTL effectors and maintaining their activated state, may be required to mediate their therapeutic effects. Since Th cells are described as regulators of protective immunity (11-14) this raised the question whether such cells are involved in survival of effector CD8+ cells and the nature of survival signals.

To address these questions, we analyzed the effects of cells stimulated with the HER-2 peptide G89(777-789) (G89-primed, G89-PR) and of the peptide G89 in induction, survival, and expression of lytic function of CTL specific for the HER-2 CTL epitope E75 (369-377) (15). G89 was found in our recent studies to induce proliferation by CD4+ cells from PBMC of breast cancer patients with no evidence of disease and of lymphocytes from lymph nodes which contained breast metastases (15, 16). E75 was identified by our previous studies to be frequently recognized by ovarian and breast tumor-reactive CTL (17, 18) and appears to be the immunodominant CTL epitope of HER-2 in the HLA-A2 system (19). E75 is endogenously processed and presented by tumor cells naturally overexpressing HER-2 or after viral transduction (20-22). Endogenous presentation of E75 makes it an important target of anti-tumor CTL. E75 easily induces IFN-γ secretion in the majority of donors, but infrequently activates lytic function of CD8+ cells (23-25). G89 is also

Abbreviations: No peptide, NP; Macrophages, MΦ; mean channel fluorescence, MCF; DC, dendritic cells; Primed, PR; lytic units, LU; anti, α.

Correspondence to: Constantin G. Ioannides, Ph.D, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 440, Houston, TX 77030, USA. Tel: 713-796-2849, Fax: 713-792-3575.

Key Words: CTL, cytokines, HER-2, helper effects, survival.

0250-7005/2002 $2.00+.40

processed and presented by APC (15). We rationalized that if G89 has helper ability it will provide the additional help needed for CTL induction and survival.

We found that G89-PR cells are involved in the activation and survival of CTL induced by E75. In addition to G89-PR cells, G89 antigen had a modulatory effect in survival and maintenance of the lytic effector function. Priming of T-cells with E75+ G89 had a positive effect in induction, expansion, and higher levels of CTL lytic function. In contrast, restimulation of primed T-cells with E75+G89 had an inhibitory effect on the survival of CD8+ cells and their lytic function compared with restimulation by E75 alone. These effects inversely correlated with the levels of expression of IL-2Ra on E75- stimulated, and E75+ G89-stimulated cells and with the levels of IL-10 secreted by E75-and E75+G89-stimulated cells. Our results show that G89 modulated the survival of E75-specific CTL. The "helper" effects by G89 were not simply a reflection of the increases in Th1 cytokines such as IFN-γ induced by G89, rather the induction of IFN-γ and the balance between IFN-γ and IL-10, modified by G89 differentially regulated the survival of effector CTL induced by E75.

Materials and Methods

Subjects. Experiments performed in these studies used primarily PBMC from a healthy donor which expressed both HLA-A2 and HLA-DR4 (A2, (homozygous), B7,44, DR4,15,DQ6,7.). This donor was selected because in previous studies the donor responded to priming with E75 by secretion of IFN-γ and induction of CTL activity, and also to G89 in the context of HLA-DR4 (14, 23, 24). The response to G89 was characterized by higher levels of IFN-γ than IL-4 and IL-10, suggesting that, in this context, G89 acted mainly as a Th1 epitope (14). Other donors used in these experiments for CTL induction were also HLA-A2+ and HLA-DR4+.

HER-2 peptides. The HER-2 peptides used as immunogens corresponded to the CTL epitope E75 (369-377), and the $CD4^+$ cells recognized epitopes G89(777-789), F7 (776-788) and G90 (886-898) (15,26). These peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center (Texas, USA), and purified as described. G89 and F7 are of the same length but differ by one amino acid at the N and C - termini. Similarly, the sequence of G90 has two amino acids deleted from the N-terminus and one from the C-terminus of the HER peptide F13 (884-898) found to induce proliferation of PBMC from ovarian cancer patients (26).

In vitro activation experiments. To examine the activation potential of G89 and of G89-primed cells, isolated plastic nonadherent PBMC (further referred to as PBMC) were used as responders in all experiments. The use of the whole population of PBMC as responders was preferred to the use of isolated CD4+ or CD8+ cells. Analysis in unseparated populations allows accounting for influences from other cells in this system. Plastic adherent PBMC were cultured in GM-CSF+IL-4 to generate monocyte-derived dendritic cells (DC) as we previously described (23, 24). DC were detached from the plastic on day 3, (3 days DC) using 0.5mM EDTA, aliquoted, and frozen in liquid-nitrogen. They were then thawed 3 days before their use as APC and cultured in GM-CSF+IL-4 for 3 additional days. This approach ensured that the same sample of DC was used as APC in all stimulation groups, and the time in culture and cytokine concentration was always the same. For antigen presentation, DC were pulsed with E75, and where indicated with E75+ G89, and used as stimulators of PBMC. G89-primed cells (G89-PR) were generated from the same donor in a separate experiment. Control, no peptide primed (NP-PR) were generated in parallel in the same experiment in the same conditions by incubating DC which were not pulsed with peptide with PBMC cells. After stimulation, both G89-PR and NP-PR cells were cultured in the presence of IL-2 for 1 week. Afterwards, the cells were washed, aliquoted and stored frozen in liquid nitrogen until use.

To determine the effects of G89 PR cells in the induction of CTL responses, G89-PR cells and control NP-PR cells were thawed, washed, and irradiated to block their proliferation. For antigen stimulation, 10^5 DC were pulsed with 50 μg E75 alone or together with 25 μg G89 for two hours in serum-free RPMI medium, in 1.0 ml. Afterwards, equal numbers (5×10^5) of irradiated G89-PR and NP-PR cells were added in each well followed by 2×10^6 PBMC. The FCS final concentration was then adjusted to 10% by addition of FCS in a final volume of 2.0 ml. Two days later, IL-2 at 60 IU/ml final concentration was added in each well and replaced every two days to maintain its concentration at 60 IU/ml. The same approach was used for restimulation. Responder cells primed with E75 and E75+G89, were rested for 24 hours before restimulation by replacing the RPMI medium with 10% FCS and antibiotics (complete medium) with medium without IL-2. IL-2 was added on day 2 after stimulation (48 hours later) and replaced every 2 days.

Flow-cytometry. For phenotypic analysis, cells were stained with α-CD8-FITC, αIL2R(CD25)-FITC, αCD40-PE, and CD40L- PE, (BD/Pharmingen). MHC-I, MHC-II, B7.1, and B7.2 expression on DC was quantitated by two-color analysis as described (23) using a CD13-PE as a marker for DC. Cells were stained for intracellular perforin expression and for surface expression of CD8 using the manufacturer protocol (BD/Pharmingen). Stained cells were analyzed using a flow-cytometer (EPICS-Profile Analyzer, Coulter Co., Hialeh, Fl.) with a log amplifer.

Cytokine assays. IL-12 was detected using an ELISA-kit which recognizes both p40 and the natural heterodimeric molecule. Detection of IL-2, IFN-g and IL-10 was performed using the corresponding ELISA-kits (Biosource). Since IL-4 levels in this system are generally low, ultra-sensitive IL-4 ELISA kits were used for detection of this cytokine (15).

Cytotoxicity and proliferation assays. Recognition of E75 was performed in ^{51}Cr release assays as we described (15). Calculation of lytic (LU) units was performed as described (27). One lytic unit was defined as the number of effectors from 10^7 cells which induced 20% lysis. The increase/decrease in cell numbers at stimulation with E75+G89 was determined by counting the numbers of live cells in culture in the presence of trypan blue.

Statistical analysis. A response to an antigen was defined as positive when differences in cpm values between cultures or targets that received peptide, and cultures which did not receive peptide were significant by the unpaired Student's test ($p < .05$). CTL recognition of antigen was considered significant when the % specific lysis of targets pulsed with the peptide antigen minus standard deviation (SD) was higher by 10% than lysis of targets which were not pulsed with peptide plus SD. For associations between the cytolytic activity and the cytokine production, we performed both two-factor analysis of variance and three factors analysis of variance using ANOVA (SPSS, Inc., Chicago, IL, USA).

Results

G89 synergize with E75 in induction of higher levels of IFN-γ. G89 was found to activate proliferation of $CD4^+$ cells from $HLA\text{-}DR4^+$ donors and induced a cytokine response shifted

towards Th1 type response (15-17). To address whether G89 enhanced Th1 cytokine induction, both E75 and G89 were pulsed on DC from Donor 1. The amounts of IFN-γ and IL-2 secreted were determined 72 hours later. G89 concentration was maintained constant at 20 mM, while E75 concentration was increased from 1 to 100 μM. The results in Table I show that the presence of G89 lead to a several-fold increase in the IFN-γ levels in response to increasing concentrations of E75 compared with cultures where G89 was absent, suggesting a synergistic effect with E75. This increase was paralleled by a decrease in the levels of IL-2 recovered from the same wells, suggesting that the presence of G89 increased IL-2 consumption. The levels of IL-2 induced by G89 were low since the amount of IL-2 recovered was always less than the amount added. The requirement for interaction between G89 and G89-activated cells was confirmed by the increased levels of IFN-γ when cells of the G89-specific line G89L (15) were added in the same experiment.

Table I. *G89 and G89L cells enhance IFN-γ induction by E75.*

	Added Antigen and Cytokine			Cytokine Recovered (pg/ml)	
	E75	G89	IL-2(1000 pg/ml)	IFN-γ	IL-2
1.	-	-	-	0	0
2.	-	-	+	40	650
3.	0	20	+	150	600
4.	25	0	+	85	560
5.	50	0	+	135	521
6.	1	20	+	230	509
7.	10	20	+	315	364
8.	25	20	+	425	293
9.	50	20	+	568	212
10.	100	20	+	693	164
11.	50	0+G89L cells	+	350	508
12.	50	20+G89L cells	+	1174	275

E75 and G89 values indicated in the columns are in μg/ml. The amounts of cytokines detected by ELISA are in pg/ml. G89L represents a G89-specific cell line obtained from the same donor by repeated stimulations with G89 presented on autologous PBMC (15).

Conditioning of DC with G89 leads to higher CTL activity at stimulation with E75. Interaction of CD4+ cells with peptides presented on DC was described to be essential for DC activation for induction of CTL against weak immunogens. We tested the ability of G89 to potentiate the CTL inducing ability of DC. To address this question, adherent three-days-cultured DC were pulsed with G89, E75, or G90, (14). DC-NP were used as negative controls. After 48 hours incubation with nonadherent PBMC, in the absence of IL-2 and IL-12, the primed cells were removed. DC were then rested for 72 hours to allow dissociation of previously pulsed Ag, then used to present E75 to PBMC primed with G89, G90, E75 or NP. The results of CTL assays (Figure 1A) show that G89-conditioned DC significantly increased the lytic response to E75 at restimulation with E75. G89-conditioning of DC increased the number of E75-specific LU by 100% compared with NP-conditioning. Furthermore, E75-specific LU induced by the G89-> E75-stimulation were 60% higher than E75-specific LU induced by the E75->E75 and G90 → E75 stimulations. This experiment was repeated with another donor and the results were confirmed (not shown). Thus, recognition of G89 on DC by unstimulated PBMC increased the CTL stimulatory ability of DC.

Recognition of G89 on DC lead to up-regulation of HLA-A2, HLA-DR and CD40. To address the question of conditioning effects induced by G89-recognition on DC, we determined the levels of surface antigen expression and the levels of IL-12 produced by DC. The conditioning effects of G89 on DC consisted in up-regulation of HLA-A2, HLA-DR and CD40, and induction of higher levels of IL-12 within 48 hours compared with G90 and E75, but not in rapid up-regulation of B7.1 and B7.2. B7.1/2 up-regulation was weak and detectable only on day 5 (not shown). It should be mentioned that the increase in MHC-I and MHC-II on DC was significantly higher than the increase in CD40 expression. Interaction of G89-PR cells with DC in the absence of G89 lead to increased CD40 levels by two-fold (from MCF=60 to MCF=130). Interaction of G89-PR cells with DC-G89 lead to another 2-fold increase (MCF=265). When DC were incubated with G89, E75, G89-PR and E75PR cells the levels of CD40 did not increase further. In contrast, interaction of E75-PR cells with DC-E75 led to a smaller increase (MCF=160) (Figure 1B and 1C). This suggested that most of CD40 up-regulation on DC was induced by recognition of G89 by G89 PR cells. To address whether the increased IFN-g induction was due to increased IL-12 induction, we determined the levels of IL-12 in response to G89, G90 and negative control (NP) stimulated DC. The results are shown in Table II. All antigens including tetanous toxoid induced IL-12 in DC. E75 and G89 induced similar levels of IL-12. The results also show additive effects of G89 and E75 in inducing higher levels of IL-12.

The presence of G89-PR cells during restimulation with E75 increased the survival of E75-PR cells. To evaluate the role of G89 and G89-primed cells in induction of an effector CTL

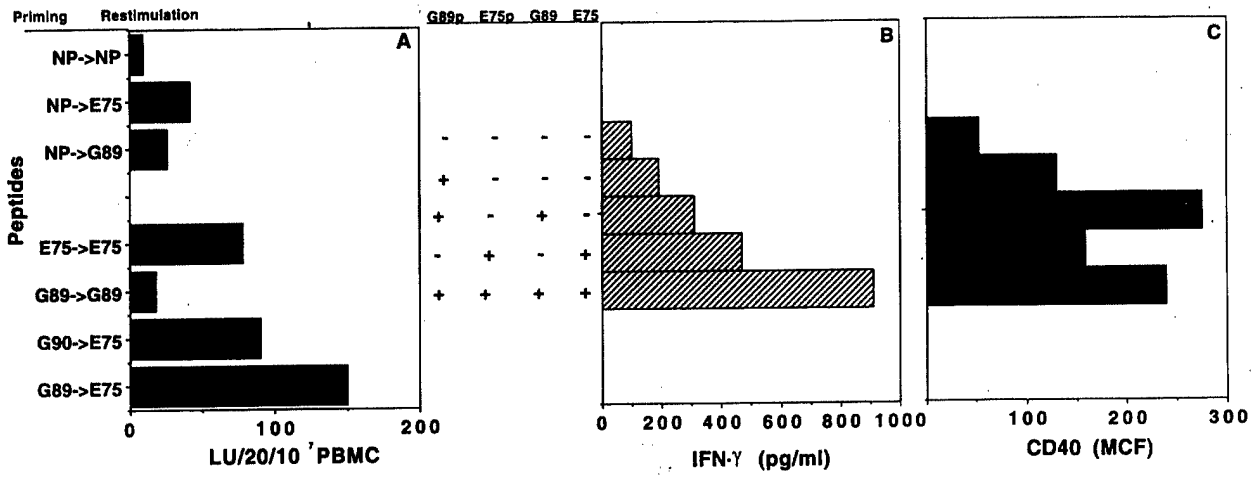


Figure 1(A). *DC "conditioning" with G89 leads to higher levels of E75-specific CTL activity at presentation of E75 than conditioning with the CTL epitope E75, or the control epitope G90 or in the absence of peptide (NP). CTL activity was determined in a 5 hours CTL assay. LU were calculated as described in Materials and Methods and are expressed per 107 PBMC. The number of $CD8^+$ cells was not determined. (B,C) Recognition of G89 on DC by G89 stimulated cells (G89-PR) up-regulated CD40 Ag expression. E75 and G89 synergized in up-regulation of IFN-γ induction. G89-PR and E75-PR indicate that T cells from DR4+ donor were primed (p) with G89 and E75, respectively. Supernatants were collected and the levels of IFN-γ, (A) and CD40 expression (B) were determined 20 hours later.*

response *in vitro* vaccinations were performed, using as helpers G89-PR cells. G89-PR cells were obtained by stimulation of PBMC with G89 followed by expansion in IL-2. As control, we used NP-PR cells generated in the same way as G89-PR cells. To determine the effects of G89-PR cells at the CTL induction phase, G89-PR and NP-PR cells were first irradiated to ensure that they would not proliferate further during stimulation with G89 or E75. Thus, contribution of G89-PR cells was limited to effects mediated by receptor interactions and existent (preformed) cytokine secretion. Experiments were performed using PMBC as responders. The reason for this approach was that the results would reflect the effects of interactions of surface antigens and cytokines in the entire population, including cross-reactivity between other HLA-DR molecules which may bind G89 and T cells. Thus the results would not be biased by addition of CD8+ and CD4+ cells, in reconstitution experiments to modify the equilibrium between cells in a population.

To determine whether G89 interfered with E75-specific CTL activation, experiments were performed both in the absence of G89 and in its presence. To define the effects of G89-PR cells, the activation experiments were performed in the presence of G89-PR or NP-PR cells. This lead to four activation groups: (1) DC+E75+PBMC+G89-PR cells; (2) DC+(E75+G89)+BMC+G89-PR cells. (3) DC+E75+ PBMC+NP-PR cells. (4) DC + (E75+G89)+PBMC+NP-PR cells (Table III).

A part of Ag-stimulated CTL usually enter the death phase after restimulation. Induction of the death phase affects not only the Ag-specific T cells but also other lymphocytes which became susceptible to apoptosis following FasL (CD95L) interaction with Fas on other lymphocytes in the absence of proper TCR engagement. We wished to examine first, whether induction of E75-specific lytic effectors was enhanced by the presence of G89 and of G89-PR cells. Second we wanted to elucidate whether the presence of G89-PR cells increased survival of E75-specific lytic effectors.

To address these questions, equal numbers of cells from each of the Groups 1-4 were restimulated with the same amounts of E75+ G89 used at priming. Equal numbers of irradiated G89-PR and NP-PR cells were added in the activation cultures. To address the effects of G89-PR cells and of G89 on CTL survival, cells from Groups 1-4 were maintained in culture, in the presence of IL-2 for 30 days. The numbers of viable cells were determined every 4 days. Cells increase in numbers until day 20, then their numbers started to decline. The results in Figure 2A and 2B show that the numbers of viable cells in Groups 3 and 4 declined faster than the numbers of viable cells in Groups 1 and 2. The differences between the number of live cells in these two groups were significant on day 27 (66-70% *vs.* 43-44%); by day 31 most cells in the Groups 3 and 4 had died compared with the Groups 1 and 2. This suggested that the presence of G89-PR cells during restimulation extended survival of E75 and E75+G89 stimulated cells compared with NP-PR cells.

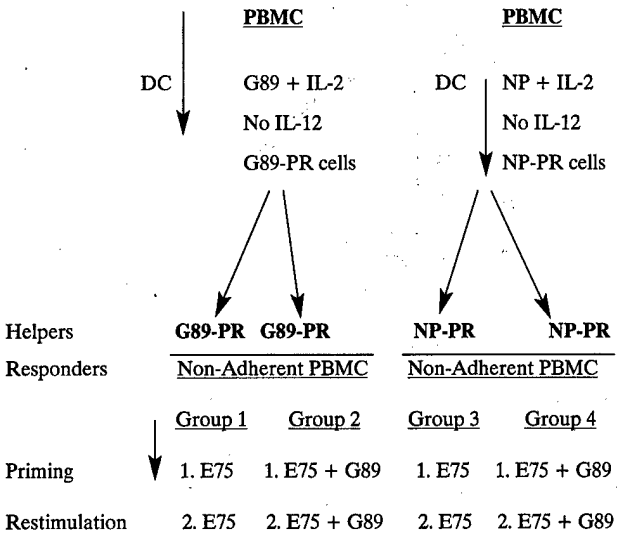
To address whether there were differences between the numbers of $CD8^+$ cells in these groups, we determined the CD8 and perforin expression on day 27 in the same experiment, when the differences in numbers between live cells in Groups 1 and 2 (containing G89-PR cells) and Groups

Table II. *Synergistic effects of E75 and G89 in IL-12 induction in dendritic cells.*

	Cells		Ag	Ab	IL-12 (pg/ml)
	G89-PR	DC			
1.	+	-	-		0
2.	-	+	-		20
3.	+	+	-		460
4.	+	+	E75		700
5.	+	+	G89		680
6.	+	+	G90		520
7.	+	+	TT		580
8.	+	+	E75 + G89	mIgG	1480
9.	+	+	E75 + G89	BB7.2	644
10.	+	+	E75 + G89	L243	773
11.	+	+	E75 + G89	aCD40L	33

G89-PR cells were generated by priming with G89 presented on autologous DC. Autologous DC not previously exposed to Ag were used for re-stimulation. IL-12 was detected 48 hours after stimulation monoclonal antibodies. BB7.2, L243, and αCD40L were used to block HLA-A2, HLA-DR, and CD40:-CD40L interactions. A murine myeloma protein (MOPC-1) was used as control murine IgG (mIgG).

Table III. *Stimulation groups used to determine the helper effects of G89 and of G89-PR cells.*



3 and 4 (containing NP-PR cells) were significant. The numbers of CD8+ cells were higher in Group 1 than in the other three groups (Table IV). In addition $CD8^+$ cells in Groups 1 and 2 contained a higher proportion of perforin-positive cells than the other two groups.

The initial CTL assay on day 18 showed higher lytic activity against E75 by effectors from Groups 2 and 4 compared with Groups 1 and 3. This activity positively correlated with the presence of G89 during restimulation, suggesting that either a higher number of E75-specific CTL were present in Groups 2 and 4, or these were more activated than the CTL in Groups 1 and 3. The second possibility was more likely since cells in the Group 2 showed significantly higher levels of CD25 than cells in Group 1, while cells in Group 4 showed higher levels of CD25 than cells of Group 3.

To address whether the higher number of CD8+ cells in Group 1 reflected a higher number of activated E75-specific CD8+ cells surviving in Group 1, CTL assays were repeated at two E:T ratios to allow calculations of LU. The results in Figures 3A and 3B show that there was a higher number of E75-specific CTL effectors with cytolytic activity in the Group 1 than in Group 2. The specific activity for E75 of Group 1 CTL was stable, because it was evident after 20 hours incubation with targets. In contrast, the specific activity of Group 2 CTL was unstable and could not be detected in the 20-hours CTL assay (Figures 3C and 3D). The differences between the two groups were significant when lytic units were calculated in relation to the numbers of CD8+ cells (Table IV). $LU/20/10^7$ $CD8^+$ cells against E75 were 630 in Group 1 and 223 in Group 2. In contrast LU/20/107 CD8+ cells against T2-NP (0 μg E75) were 218 and 224, respectively. This ratio LU(E75): LU(0) was 2.92 in Group 1 and 0.99 in Group 2. Taking into consideration that Group 2 CTL showed significantly higher lytic activity on day 14 than Group 1 CTL (not shown), these results indicate that the loss of E75-specific CTL effectors was higher in Group 2 than in Group 1. Thus, the presence of G89-PR cells at restimulation increased the overall survival of effector CTL compared with NP-PR cells.

To address whether differences in survival of E75-specific CTL effectors between Groups 1 and 2 reflected differences in cytokine levels in the environment due to G89, the levels of IFN-γ and IL-10 were determined in parallel during restimulation for 4 consecutive days. The results in Figures 4A and 4B show that the levels of IFN-γ were not significantly different between Groups 1 and 2. In contrast, the levels of IL-10 were higher in Group 1, which were not restimulated with G89 than in Group 2 which were restimulated with G89. The kinetics of IL-10 secretion was different in the two groups. Group 1 cells showed a tendency for exponential increase in the IL-10 levels, while Group 2 cells showed a rather linear increase in the IL-10 levels. Thus, at restimulation, G89 continued to elicit a Th1 pattern of cytokine secretion.

To address whether the effects of G89 reflected as cytokine secretion correlated with the lytic activity or days after stimulation we performed statistical analysis. The results showed a positive correlation between E75-specific lysis by Group 1 and the levels of IL-10, but not with the levels of

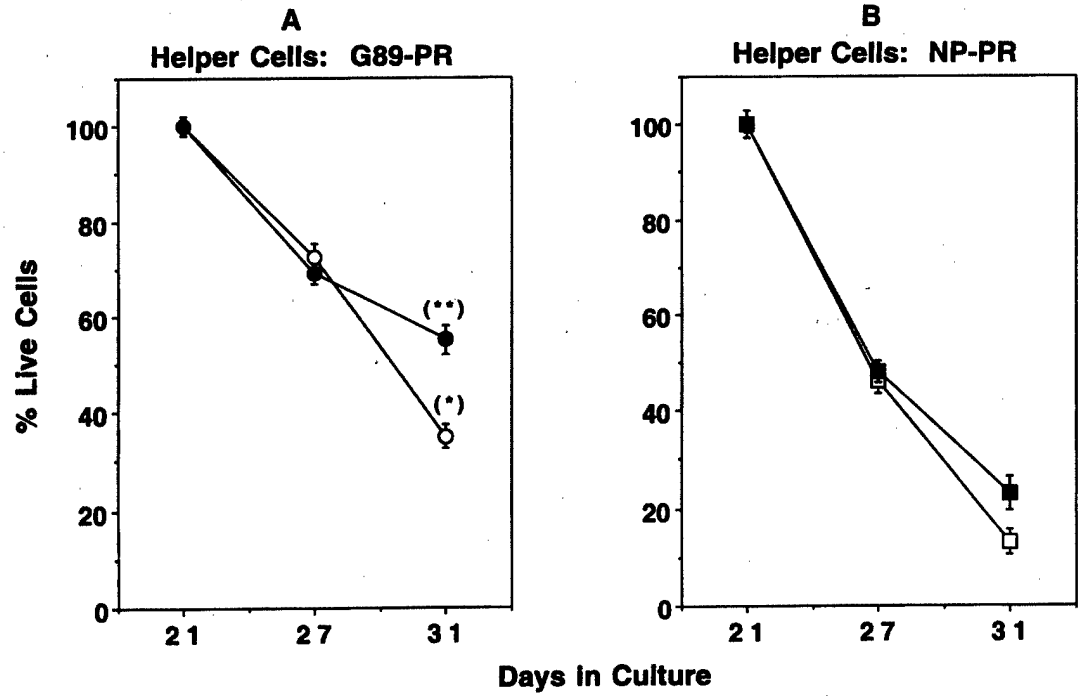


Figure 2A, B. *(A) Increased survival of cells primed and restimulated with E75 in cultures containing irradiated G89-PR cells, comparing with, (B) cells from cultures stimulated with E75 or E75+G89 containing NP-PR cells as helpers cells. Results from one experiment representative of three independently performed determinations are shown. On day 21 cells in each well exceeded 10^6. (●) Group 1, (○) Group 2, (■) Group 3, (□) Group 4, (**) indicate that the number of surviving cells in Group 1 was significantly higher than the number of surviving cells in Groups 3 and 4. (*) indicate that the number of surviving cells in Group 2 was significantly higher than the number of surviving cells in Group 4.*

IFN-γ. This suggested that extended CTL survival might be a consequence of the IL-10 produced in the environment when G89 was absent. The levels of IL-10 and IFN-γ were analyzed using two-factor analysis of variance with day after stimulation and composition: Group 1 (E75) *vs* Group 2 (E75+G89) (The two impact factors). The factor day stimulation was highly significant ($p>0.001$) for both IL-10 and IFN-γ. For IFN-γ secretion, Groups 1 and 2 were not significantly different ($p=0.063$). For IL-10, however, the differences between groups were highly significantly ($p<0.001$) with Group 1 (E75 alone) significantly higher than Group 2 (E75+G89) over all days (Figure 4B). Thus in this system G89 impacted on IL-10 induction, more than in IFN-γ induction.

To analyze the effects of E:T Ratio, Ag expression on target, and Ag used for stimulation of each group on cytolysis, we subjected the data to a three-factor analysis of variance. In this case the factors Ag expression and composition of the stimulation groups were highly significant ($p<0.001$). The E:T ratio was also significant ($p=0.0120$). There was also a significant difference in lysis between the presence of E75 on target and stimulation group ($p=0.004$). This implied that recognition of T2-E75 by Group 1 at E:T=26 was significantly higher than any of the other three effector-target combinations ($p<0.001$). The other three combinations were all the same ($p=0.81$) using a one-factor analysis of variance and the Tukey Honestly Significant Difference multiple range test. These results suggest that restimulation of E75-reactive

Table IV. *CD8 and perforin expression in cells of the stimulation Groups 1-4.*

	Groups (% Positive Cells)			
	1	2	3	4
CD8+	24.4	15.1	12.2	8.1
CD8BR	8.3	8.2	6.0	6.7
CD8+ Perf+	11.9	10.4	6.2	5.3

*Immunofluorescence was performed on lymphocytes from stimulation cultures, Groups 1-4. Intracellular staining for perforin was performed following the manufacturer's instructions. Cells were stained with α-CD8-FITC followed by α-Perforin PE. The percentages of positive cells are shown. BR indicated CD8 bright cells with high levels of expression of CD8.

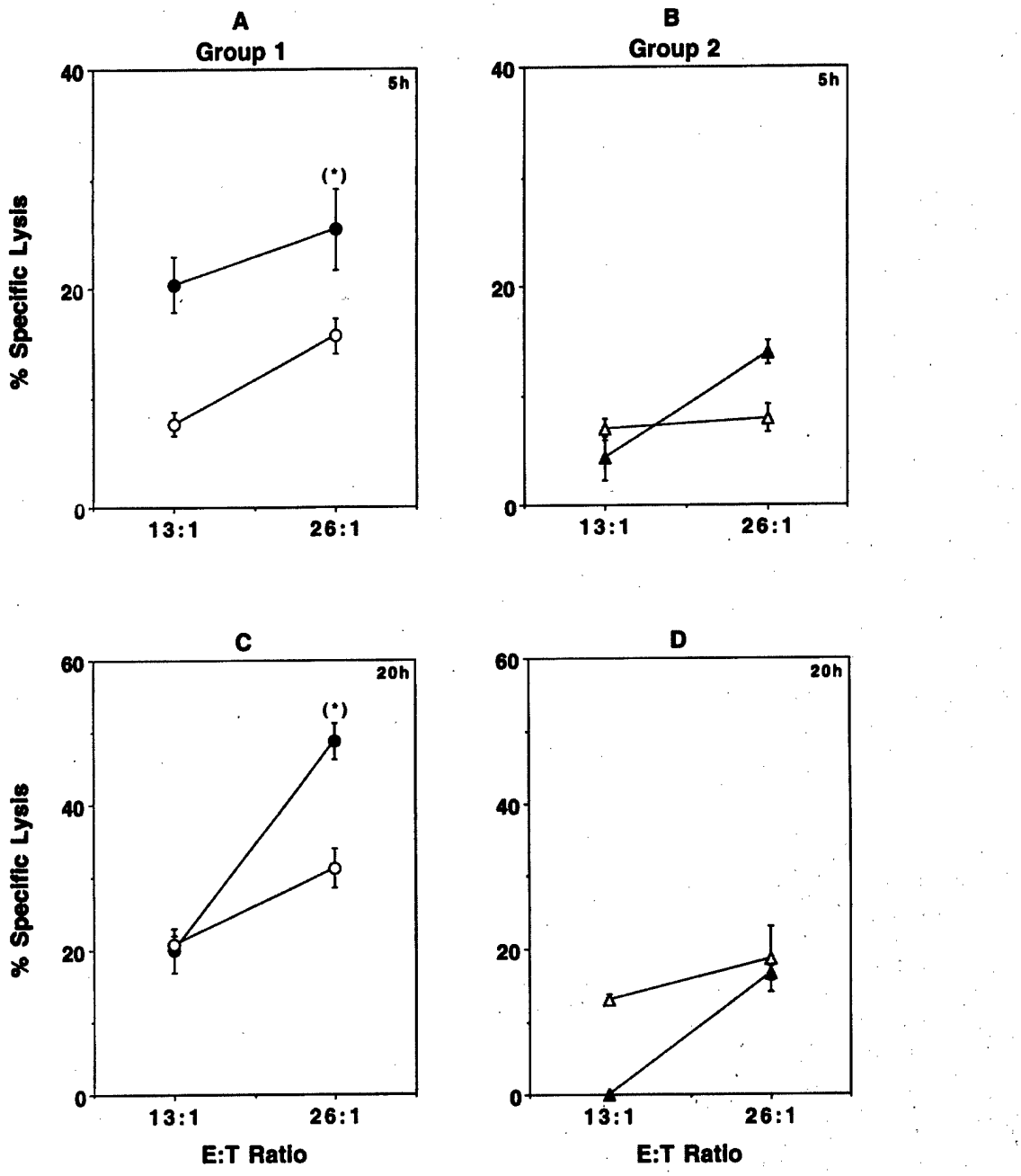


Figure 3A, B, C, D. *Cytolytic activity of day 27 E75-stimulated (Group 1) and E75+G89-stimulated (Group 2) PBMC. (A, B) 4-hours CTL assay; (C, D) 20-hours CTL assay. T2 cells were pulsed with E75 at 10 μg/ml (•, ▲), or they were not pulsed with peptide NP (○, ▲). (*) indicate that lysis of E75 pulsed T2 by Group 1 CTL was significantly higher than lysis of targets not pulsed with peptide.*

cells with DC-E75 in the presence of G89-PR cells but without G89 was the best approach for induction of Ag-specific long-lived CTL.

Discussion

Our previous studies identified the HER-2 peptide G89 (778-789) as an antigen which activated T-lymphocytes from individuals expressing HLA-DR4. In this report, we document that G89 and G89-primed cells can modulate the ability of CD8+ cells specific for a CTL epitope of HER-2, E75(369-377), to elicit lytic function and to survive in culture. Activation of cytolytic function was dependent on the presence of G89 during stimulation with E75 and/or the prior "conditioning" of DC by T cells recognizing G89. Survival of CD8+ cytolytic effectors was dependent on the presence of

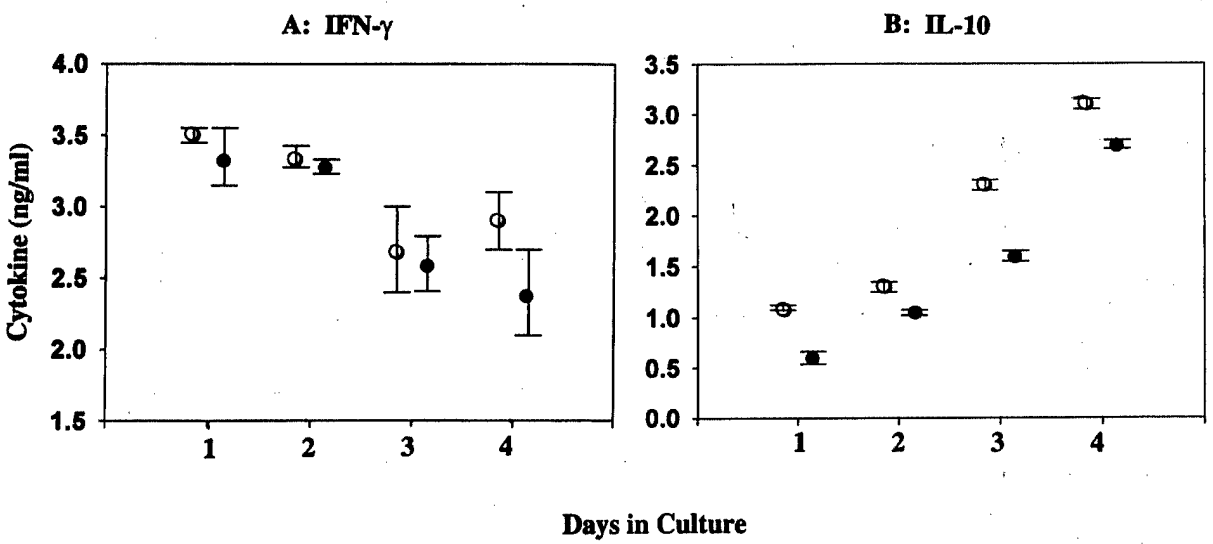


Figure 4A, B. *Kinetics of IFN-γ (A) and IL-10 (B) response at re-stimulation by cells of Group 1 (E75-stimulated) (○) and Group 2 (E75+G89-stimulated) (●). Irradiated G89-PR cells were present in both groups.*

G89-primed cells both at priming and restimulation with E75. CD8+ CTL survival decreased when the "helper" Ag (G89) was present during restimulation. The decrease in survival appeared to correlate with the reduced levels of IL-10 secreted by cells stimulated by E75+G89 compared with cells restimulated with E75 alone. Since in the HLA-DR4 system G89 acts as a Th1 epitope, overactivation of the IFN-g production appeared to inhibit long-term survival of E75-specific CTL.

G89, G89-PR and G89-reactive T cells can be an extremely potent tool to activate CTL responses to tumors in defined MHC-II systems and modulate the survival of effector CTL, even if the CTL epitope it is not precisely known; G89 was identified based on its ability to activate proliferative responses of PBMC from breast cancer patients without evidence of disease as well as of PBMC from healthy donors (15). In many instances, the magnitude of proliferative responses to the overlapped epitope F7 and G89 was similar suggesting that the core of the active antigen reside in the 11-mer sequence: SPYVSRLLGICL. Recent studies in our laboratory demonstrated that G89 can activate proliferation and Th1 cytokine responses by lymphocytes from lymph nodes of breast cancer patients with and without metastases (16) and T cells from peripheral blood of patients with ductal cell carcinoma (DCIS) *in situ* (Murray, JL, Gillogly, M, manuscript in preparation). This also suggested that G89-primed or cross-reactive T cells are present not only during disease but also in healthy individuals and may play a regulatory role in generating tumor-specific CD8+ T cell response.

The precise nature of the role of CD4+ cells in mediating anti-tumor effector functions is still unclear: One group of studies demonstrated that CD4+ T cells provide help for CD8+ cells during priming to achieve full activation of effector function (reviewed in 7, 28). This can be manifested by engagement of the CD40 on the APC by CD40L on activated CD4+ cells. This leads to up-regulation of CD40, secretion of IL-12 and up-regulation of MHC-1 and MHC-II molecules with consequent Ag-presentation at higher density. Other studies suggested that the helper effects of CD4+ cells are due to their ability to provide cytokines which can activate tolerized/anergized CD8+ (6,29). Possible pathways of help for CD8+CTL by CD4+ cells are either through the IL-2°IL2R-α pathway which leads to enhanced CD8+ cells clonal expansion and perforin synthesis or through the IL-12°IFN-γ pathway which enhance the cytokine effector function of CD8+ CTL.

Our results showed that G89 induced IL-12 in DC, and potentiated induction of IFN-γ when E75 was used as immunogen. G89 was a weak inducer of IL-2. Furthermore, G89-PR cells induced higher levels of expression of CD40 on DC. E75+E75-PR cells cannot up-regulate CD40 at higher levels than the ones induced by G89+G89-PR cells. These results together indicate that G89 is a helper epitope for DC "conditioning" and for induction of epitope-specific CTL in the HLA-A2 and HLA-DR4 system.

Results in some models showed that Ag can induce anti-tumor responses without $CD4^+$ cells help (30); in other models the CD4-independent induction of $CD8^+$ CTL was accomplished through the B7-CD28 pathway of costimulation

(31). However, in other models $CD4^+$ cells secreted cytokines which rescued $CD8^+$ cells from anergy induced by Ag plus B7.1 costimulation (32). More recently a role of $CD4^+$ cells in survival of $CD8^+$ CTL and maintenance of their CTL effector function has been reported (33-35). These CTL did not require CD4 help during the induction phase.

After the peak expansion, protection of $CD8^+$ cells from death induced by restimulation required the presence of G89-primed cells, but not of G89 antigen. Therefore, for "death protected"CTL, another possible pathway of help by $CD4^+$ cells, is their ability to provide cytokines which can attenuate the activation of apoptotic pathways. The "death protection" by G89-primed cells, was associated with a lower Th1/Th2 cytokine ratio than the one induced by restimulation with G89. These results support a strong, cytokine (IL-10)-mediated, survival effect by G89-primed cells in the absence of G89.

IL-10 acts through a heterodimeric membrane receptor made of two subunits IL-10R1 and IL-10R2. While immature DC express comparable levels of the IL-10 binding receptor (IL10R1) and the signaling subunits (IL-10R2), the ratio between subunits change in DC matured by various approaches (36). The IFN-γ R1 and IL-10 R1 share many similarities in their structure, and the corresponding cytokines may interfere with signaling against each other (36). The involvement of IL-10 in CTL survival and maintenance of cytolytic function was unexpected. The protective effects of IL-10 were not mediated in a context where IFN-γ was absent, rather the levels of IFN-γ were initially higher than the levels of IL-10, then they were similar, and over time became lower than the levels of IL-10. This suggested a possible mechanism of "interference" by IL-10 with activation of death pathways by IFN-γ. Thus, it is possible that the IL-10 effects consisted at least in part in attenuation of IFN-γ signaling to Jak1+Jak2→STAT1, or to Jak1+Tyk2→STAT3 which connect with the IFN response elements.

This possibility is supported by recent reports on survival of other type of cells by differential production of Th1 and Th2 cytokines (37,38). Proapoptotic effects induced by IFN-γ were mediated through CD95-up-regulation and caspase activation, while IL-10 induced death protection mediated by potent up-regulation of cFLIP and Bc-XL (37). Other reports indicated a potentiating role for IL-10 in induction (or maintenance) of anti-tumor cytolytic effectors (39). The role of IL-10 as modulator of cytolytic responses only recently started to be investigated. Although IL-10 has an inhibitory role on activation of DC for mediation of type 1 responses and on antigen - and mitogen - induced activation and proliferation (40,41), it appears to synergize with IL-1 and recombinant nonreplicating poxvivus-based vaccine in enhancing virus - specific cytolytic activity (42). These effects may be due to its "attenuating" ability for apoptosis induction in activated T cells.

The results of this study may have implications for the design of novel cancer vaccination strategies. One strategy that emerges from these studies is to first prime the vaccine recipient for activation of Th1 CD4+ cells. This "help" may be provided by a Th1 antigen distinct from the tumor Ag (*e.g.* influenza). Second, to vaccinate with the Th1 epitope together with the CTL epitope. This should amplify Th1 cytokines induction and provide the necessary help for CTL expansion. Third, to boost the anti-tumor responses using only the CTL epitope, to avoid overstimulation of the Th1 response with subsequent CTL death. Modulation of cytokine responses can also be accomplished using low concentrations (pM) of IL-12 as a proinflammatory cytokine at priming with Th1 epitope, and of IL-10 (or IL-4) after boosting with the CTL epitope. Regulation of CTL responses to tumor by induction of $CD4^+$ regulatory cells is of particular interest since it may overcome the two fundamental problems that hamper vaccine therapies: expansion of functional tumor-reactive CTL, and protection of these CTL from death by overstimulation.

Acknowledgements

We thank Mr. Scott Kelly for his technical assistance and Mr. Brett Anderson for his assistance with flow cyometry. We also thank the Blood Bank personnel who volunteered to donate blood for these studies. This work was supported in part by the grant DAMD 17-97-I7098. Peptide synthesis was supported in part by the core grant CA16672.

References

1 Bour H, Horvath C, Lurquin C, Cerottini JC and MacDonald HR: Differential requirement for CD4 help in the development of an antigen-specific CD8+ T cell response depending on the route of immunization. J Immunol *160:* 5522-5529, 1998.

2 Ostankovitch M, Le Gal FA, Connan F, Chassin D, Choppin J and Gillet JG: Generation of Melan-A/MART-1-specific CD8+ cytotoxic T lymphocytes from human naïve precursors: helper effect requirement for efficient primary cytotoxic T lymphocyte induction *in vitro.* Int J Cancer *72:* 987-994, 1997.

3 Surman DR, Dudley ME, Overwijk WW and Restifo NP: Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. J. Immunol. *164:* 562-565, 2000.

4 Shrikant P, Khorutus A and Mescher MF: CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell-and IL-2-dependent mechanism. Immunity *11:* 483-493, 1999.

5 Baxevanis CN, Voutsas IF, Tsitsilonis OE, Gritzapis AD, Sotiriadou R and Papamichail M: Tumor-specific CD4+ T lymphocytes from cancer patients are required for optimal induction of cytotoxic T cells against the autologous tumor. J Immunol *164:* 3902-3912, 2000.

6 Lu Z, Yuan L, Zhou X, Sotomayor E, Levitsky HI and Pardoll DM: CD40-independent pathways of T cell help for priming of CD8(+) cytotoxic T lymphocytes. J Exp Med *191:* 541-550, 2000.

7 Toes REM, Schoenberge SP, van der Voort EIH, Offringa R and Melief CJM: CD40-CD40 Ligand interactions and their role in cytotoxic T lymphocyte priming and anti-tumor immunity. [Review]. Semin Immunol *10:* 443-448, 1998.

8 Panus JF, McHeyzer-Williams LJ and J McHeyzer-Williams G: Antigen-specific T helper cell function: Differential cytokine expression in primary and memory responses. J Exp Med *192:* 1301-1316, 2000.

9 Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D and Levitsky H: The central role of CD4+ T cells in the antitumor immune response. J Exp Med *188:* 2357-2368, 1998.

10 Whitmire JK and Ahmed R: Costimulation in antiviral immunity: Differential requirements for CD4+ T cell responses. Curr Opin Immunol *12:* 448-455, 2000

11. Lopex-Dias de Cerio AL, Casares N, Lasarte JJ, Sarobe P, Perez-Mediavilla, LA Ruiz M, Prieto J and Borras-Cuesta F: T(h)1 but not T(h)0 cell help is efficient to induce cytotoxic T lymphocytes by immunization with short synthetic peptides. Int Immunol *11:* 2025-2034, 1999.

12 Shimizu J, Yamazaki S and Sakaguchi S: Induction of tumor immunity by removing CD25 + CD4+ cells: a common basis between tumor immunity and autoimmunity. J Immunol *163:* 5211-5218, 1999.

13 Wild J, Grusby MJ, Schirmbeck R and Reimann J: Priming MHC-I-restricted cytotoxic T lymphocyte responses to exogenous hepatitis B surface antigen is CD4+ T cell-dependent. J Immunol *163:* 1880-1887, 1999.

14 Cheng TY, Wu JT and Lin RH: Induction of tumor-specific T cell response by cognating tumor cells with foreign antigen-primed Th cells. Int Immunol *10:* 1397-1406, 1998.

15 Tuttle TM, Anderson BW, Thompson WE, Lee JE, Sahin A, Smith TL, Grabstein KH, Wharton JT, Ioannides CG and Murray JL: Proliferative and cytokine responses to Class II HER-2/neu-associated peptides in breast cancer patients. Clin Cancer Res *4:* 2015-2024, 1998.

16 Kuerer HM, Peoples GE, Sahin AA, Murray JL, Singletary E, Castilleja A, Hunt KK, Gershenson DM and Ioannides CG: Axillary lymph node cellular immune response to HER-2/neu peptides in patients with carcinoma of the breast. J Interf Cytokine Research (submitted), 2002.

17 Fisk B, Blevins TL, Wharton JT and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med *181:* 2109-2117, 1995.

18 Hudson JM, Castilleja A, Murray JL, Honda T, Kudelka A, Singletary AE, Wharton JT and Ioannides CG: Growth and antigen recognition by tumor infiltrating lymphocytes from human breast cancer. J Interf Cytok Res *18:* 529-536, 1998.

19 Rongcun Y, Salazar-Onfray F, Charo J, Malmberg KJ, Evrin K, Maes H, Kono K, Hising C, Petersson M, Larsso O, Lan L, Appella E, Sette A, Celis E and Kiessling R: Identification of new HER2/neu-derived peptide that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. J Immunol *163:* 1037-1044, 1999.

20 Zum Buschenfelde CM, Nicklisch N, Rose-John S, Peschel C and Bernhard H: Generation of tumor-reactive CTL against the tumor-associated antigen HER2 using retrovirally transduced dendritic cells derived from CD34+ hemopoietic progenitor cells. J Immunol *165:* 4133-4140, 2000.

21 Fisk B, DaGue B, Seifert W, Kudelka A, Murray JL, Wharton JT and Ioannides CG: Mass-spectrometric analysis of naturally processed peptides recognized by ovarian tumor associated CD8+ CTL. Intl J Oncol *10:* 159-169, 1997.

22 Lustgarten J, Theobald M, Labadie C, LaFace D, Peterson P, Disis ML, Cheever MA and Sherman LA: Identification of Her-2/Neu CTL epitopes using double transgenic mice expressing HLA-A2.1 and human CD.8. Human Immunol *52:* 109-118, 1997.

23 Lee TV, Anderson BW, Peoples GE, Castilleja A, Murray JL, Gershenson DM and Ioannides CG: Identification of activated tumor antigen-reactive CD8+ cells in healthy individuals. Oncol Reports *7:* 455-466, 2000.

24 Anderson BW, Peoples GE, Murray JL, Gillogly MA, Gershenson DM and Ioannides CG: Peptide priming of cytolytic activity to HER-2 epitope 369-377 in healthy individuals. Clin Cancer Res *6:* 4192-4200, 2000.

25 Zaks TZ and Rosenberg SA: Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu + tumors. Cancer Res *58:* 4902-4908, 1998.

26 Fisk B, Hudson JM, Kavanagh J, Murray JL, Wharton JT, Ioannides CG and Kudelka PA: Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patient to HER-2 peptides. Anticancer Res. *17:* 45-53, 1997.

27 Ioannides CG, Platsoucas CD, Rashed S, Wharton JT, Edwards CL and Freedman RS: Tumor cytolysis by lymphocytes infiltrating ovarian malignant ascites. Cancer Res *51:* 4257-4265, 1991.

28 Clarke SR: The critical role of CD40/CD40L in the CD4-dependent generation of CD8+ T cell immunity. J Leuk Biol *67:* 607-614, (Review), 2000.

29 Ruedl C, Kopf M and Bachmann MF: CD8+ T cells mediate CD40-independent maturation of dendritic cells *in vivo.* J Exp Med *189:* 1875-1884, 1999.

30 Dalyot-Herman N, Bathe OF and Malek TR: Reversal of CD8+ T cell ignorance and induction of anti-tumor immunity by peptide-pulsed APC. J Immunol *165:* 6731-6737, 2000.

31 Zhan Y, Corbett AJ, Brady JL, Sutherland RM and Lew AM: CD4 help-independent induction of cytotoxic CD8 cells to allogeneic P815 tumor cells is absolutely dependent on costimulation. J Immunol *165:* 3612-3619, 2000.

32 Deeths MJ, Kedl RM and Mescher MF: CD8+ T cells become nonresponsive (anergic) following activation in the presence of costimulation. J Immunol *163:* 102-110, 1998.

33 Marzo AL, Kinnear BF, Lake RA, Frelinger JJ, Collins EJ, Robinson BW and Scott B: Tumor-specific CD4+ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. J Immunol *165:* 6047-6055, 2000.

34 Hu HM, Winter H, Urba WJ and Fox BA: Divergent roles for CD4+ T cells in the priming and effector /memory phases of adoptive immunotherapy. J Immunol *165:* 4246-4253, 2000.

35 Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD and Ahmed R: Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med *188:* 2205-2213, 1998.

36 Corinti S, Albanesi C, La Sala A, Pastore Saveria and Girolomoni G: Regulatory activity of autocrine IL-10 on dendritic cell functions. J Immunol *166:* 4312-4318, 2001.

37 Stassi G, Di Liberto D, Todaro M, Zeuner A, Ricci-Vitiani L and De Maria R: Control of target cell survival in thyroid autoimmunity by T helper cytokines via regulation of apoptotic proteins. Nature (Immunol.) *1:* 483-488, 2000.

38 Homann D, Holz A, Bot A, Coon B, Wolfe T, Petersen J, Dyrberg TP, Grusby MJ and von Herrath MJ: Autoreactive CD4+ T cells protect from autoimmune diabetes *via* bystander suppression using the IL-4/STAT6 pathway. Immunity *11:* 463-472, 1999.

39 Ebert EC: IL-10 enhances IL-2-induced proliferation and cytotoxicity by human intestinal lymphocytes. Clin & Exp Immunol *119:* 426-432, 2000.

40 Halak BK, Maquire Jr HC and Lattime EC: Tumor-induced interleukin-10 inhibits type 1 immune responses directed at a tumor antigen as well as a non-tumor antigen present at the tumor site. Cancer Res *59:* 911-917, 1999.

41 Brossart P, Zobywalski A, Grunebach F, Behnke L, Stuhler G, Reichardt VL, Kanz L and Brugger W: Tumor necrosis factor alpha and CD40 ligand antagonize the inhibitory effects of interleukin 10 on T-cell stimulatory capacity of dendritic cells. Cancer Res *60:* 4485-4492, 2000.

42 Kaufman HL, Rao JB, Irvine KR, Bronte V, Rosenberg SA, Resitfo NP and Irvine KR: Interleukin-10 enhances the therapeutic effectiveness of a recombinant pox virus-based vaccine in an experimental murine tumor model. J Immunother *22:* 489-496, 1999.

Received January 29, 2002
Accepted March 24, 2002

Identification of activated tumor antigen-reactive CD8+ cells in healthy individuals

TOM V. LEE[1], BRETT W. ANDERSON[1], GEORGE E. PEOPLES[2,4], AGAPITO CASTILLEJA[1], JAMES L. MURRAY[3], DAVID M. GERSHENSON[1] and CONSTANTIN G. IOANNIDES[1,5]

Departments of [1]Gynecologic Oncology, [2]Surgery, [3]Bioimmunotherapy and [5]Immunology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

Received January 21, 2000; Accepted February 11, 2000

Abstract. We investigated the ability of HER-2 peptide E75, which maps an immunodominant CTL epitope for ovarian and breast tumor-associated lymphocytes (TAL), to activate effector functions in freshly isolated CD8+ cells from healthy individuals. IFN-γ was rapidly induced by E75 within 20-24 h, in five of six healthy donors, in the presence of IL-12 and was detectable as early as 6 h. The IFN-γ levels were Ag-concentration dependent. Similar results were obtained with peptides mapping CTL epitopes from two other tumor Ag: folate binding protein (FBP) and amino-enhancer of split of Notch (AES). IFN-γ was also detected, from freshly isolated, unstimulated PBMC in response to HLA-A2 matched tumors + IL-12 but not of IL-12 alone. The major source of IFN-γ were CD45RO+ CD8+ cells. Induction of IFN-γ and IL-2 from CD8+ cells and of IL-12 from dendritic cells (DC) by CD8+ cells reactive with E75 mirrored their induction by the influenza matrix peptide (M1: 58-66) in the same individual. Responses to M1 are used to define the presence of activated memory cells in healthy individuals. Compared to M1 responses E75 recognition induced 2-4-fold lower levels of IL-12 from the same APC and IFN-γ and IL-2 from the same CD8+ cells. At lower Ag concentrations the endogenous IL-12 induced by E75-reactive CD8+ cells did not reach the threshold required to co-stimulate for IFN-γ. αB7.1 synergized with E75 in increasing the overall levels of IL-2 induced within 24 h. The presence of tumor Ag-reactive activated CD8+ cells in healthy individuals may improve our understanding of the mechanisms of immunosurveillance and regulation of immune responses by tumors.

Correspondence to: Dr Constantin G. Ioannides, The University of Texas M.D. Anderson Cancer Center, Department of Gynecologic Oncology, 1515 Holcombe Blvd., Box 67, Houston, TX 77030, USA

Present address: [4]Department of Surgery, Uniformed Armed Services, University of the Health Sciences, Bethesda, MD 20814, USA

Abbreviations: TAL, tumor associated lymphocytes; α, anti; DC, dendritic cells; HER-2, HER-2/neu proto-oncogene; HS, human serum; NP, no peptide; AES, aminoenhancer of split; FBP, folate binding protein

Key words: CTL, HER-2, interferon-γ, IL-12, IL-2, CTLA-4, B7

Introduction

The recent characterization of tumor Ag recognized by CTL opened the possibility of development of Ag and epitope-specific cancer vaccines. Tumor Ag recognized by melanoma, ovarian, and breast CTL have been demonstrated to be self-proteins (1). The fact that in cancer patients, CTL-specific for these self peptides co-exist with progressive tumors, suggest that such responses can be primed *in vivo*, but either CTL do not expand to the numbers required to mediate an effective response, or they expand but they are not functional in their state *in vivo*. This raise the question whether tumor occurrence induced an inefficient CTL response, or subsequent to tumor progression, tumor or host derived factors suppressed a protective pre-existent CTL response, established during the life of the patient. An alternative possibility is that tumor progression coincided with exhaustion of the protective CTL response.

The information regarding the presence and functionality of activated effector and memory tumor Ag reactive CD8+ cells in healthy individuals is limited. The fact that detection of *ex vivo* activated cytolytic effctors require repeated stimulation with Ag, mitogens and cytokines, did not allow to assess the functional phenotype of these cells (2,3) However, in one extensive study, specific cytolysis against MART-1 (27-35) was detected significantly earlier in melanoma patients than in health donors indicating a lower level of sensitization of healthy individuals by tumor Ag than of cancer patients (4). This also suggested that either different tolerance mechanisms are operative in maintaining the unresponsive state in healthy donors compared with cancer patients or these cells, in healthy individuals, are hyporesponsive to Ag in terms of cytolytic activity and possibly proliferation compared with cancer patients.

The possibility that these cells are hyporesponsive to Ag suggests that some tumor Ag may be partial or weak agonists for activation of a specific functions. If this is the case, in the presence of high Ag levels originated from tumor cells, these cells may expand, while in healthy donors the low endogenous Ag levels may be unable to expand these cells. However, the low Ag levels may activate effector functions different from cytolysis. Since vaccination strategies aim to induce long lived memory against tumors, the presence of activated effectors in healthy donors may be significant for understanding tumor immunosurveillance and its relevance to protective tumor immunity (5).

To address the presence of activated effectors in the absence of disease, and establish the ability of tumor Ag to activate the effector functions of $CD8^+$ cells, we investigated the ability of peptide E75, HER-2 (369-377) (6), to stimulate cytokine induction within less than 24 h in freshly isolated, peripheral blood $CD8^+$ cells from healthy individuals, when pulsed on $CD13^+$ $CD14^-$ DC (DC-E75). E75 is not only recognized by *in vivo* generated ovarian CTL-TAL, but *in vitro* E75 restimulated, *ex vivo* primed T-cells from cancer patients mediate specific tumor lysis (7,8). We used as antigen presenting cells (APC) autologous $CD14^+$ derived dendritic cells (DC) because of their reported higher presenting ability than other APC.

Since the frequency of $CD8^+$ cells bearing TCR capable of recognizing tumor Ag may be low and primary stimulation of PBMC with most tumor Ag, including HER-2 is inefficient in induction of detectable specific cytolytic effectors we focused our analysis on cytokine induction by peptides corresponding to CTL epitopes from HER-2 (6-9) and the newly identified tumor Ag: folate-binding protein (FBP) and amino-enhancer of split (AES) in PBMC from healthy donors (10,11). This analysis can detect response patterns to Ag, the presence of activated memory effectors (12), the ability of the tumor peptide to induce a type 1 (inflammatory response), and the requirements for co-stimulation for amplification of this response. The advantage of this model system is that the effects of defined tumor Ag on activation of peripheral T-cells from healthy donors are not perturbed, or polarized by prior *in vitro* culture with Ag + cytokines. This also adds a component of physiologic relevance to the activation pathways investigated since Ag + cytokine induced activation may be critical to the patient's response to tumors.

We rationalized that the patterns of response to Ag should be indicative of the nature of responders in the population as follows: If the Ag targets naive cells, they will respond if costimulatory receptors are present on APC and bind to their appropriate ligands. An effector response by cytokines will be observed after progression through the cell cycle and 2-4 divisions (i.e., 30-40 h) (13). This response will be inhibited by antibodies to costimulatory molecules such as B7.1/B7.2. If Ag induces tolerance, then naive cells may express a partial response at priming but they will not develop a response at restimulation (14). If activated effectors are present and are tolerized/anergized by exposure to Ag in the absence of costimulation, they will be unable to respond to the cognate/crossreactive stimulus that was initially effective for their activation. Analysis of the cytokine response can distinguish whether Ag induces anergy (characterized by minimal IL-2 secretion) or cytokine mediated immunosuppression due to high levels of IL-10 (12-14). In contrast, if activated effectors are present, they will immediately or rapidly respond to Ag by cytokine secretion without requiring division (15). In this case costimulation through surface receptors may have an enhancing/stabilizing effect on some responses (e.g., CD28 on IL-12R expression) and a regulatory effect on other responses (e.g., proliferation) due to B7 ligation by negative signaling receptors (e.g., CTLA-4) present on activated cells (16). Thus characterization of the patterns of reactivity of PBMC to tumor Ag can provide an answer to these questions.

DC-E75 stimulated high and rapid Ag specific IFN-γ, secretion by these $CD8^+$ cells in most donors in the presence of IL-12. DC-E75 also induced IL-2 in these cells while αB7.1 enhanced IL-2 production. The tumor Ag reactive cells were below the affinity threshold for triggering IL-12 production by DC. Similarly the inhibition of IL-2 induction by B7-CTLA-4 interaction helps maintaining these cells in a functionally competent but low reactive state. The primary DC-E75 stimulation even in the presence of αB7.1, or α-CTLA-4 enhanced weakly T-cell proliferation and did not enhance specific cytotoxicity. Our results indicate that activated effector cells reactive with E75 and other tumor Ag are frequently present in healthy donors. These cells appear to be neither Ag ignorant nor functionally anergized, but the tumor Ag acts as a weak/partial agonist by selectively inducing only a subset of $CD8^+$ effector functions.

Materials and methods

Cells, antibodies and cytokines. HLA-$A2^+$ PBMC were obtained from healthy volunteers from the Blood Bank of M.D. Anderson Cancer Center. The HLA phenotypes of the donors used in this study are as follows: donor 1 (A2, B7, 44), donor 2 (A2, 33, B40, 44), donor 3 (A2, 33, B41, 81), donor 4 (A1, 2, B27, 44), donor 5 (A1, 2 B44, 57, Cw5, 6), donor 6 (A2, 31, B35, 44, Cw4, w5). T2-cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (9). mAb to CD3, CD4, CD8 (Ortho), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and B7.2 (CD80 and CD86, Calbiochem), ICAM-1 (ICAM-1 (CD54, Calbiochem), CD40L (Ancell, Bayport, MN), HLA-A2 (clone BB7.2, ATCC), and MHC-II (L243, Dako Corp., Carpintera, CA) were used as unconjugated FITC or PE conjugated. Anti-CTLA-4 was a kind gift from Dr Peter Linsley (Bristol-Myers, Seattle, WA). mAb specific for IL-12, IFN-γ and isotype controls were obtained from Pharmingen. The following cytokines were used: GM-CSF (Immunex Corp., Washington, DC), specific activity 1.25×10^7 CFU/250 mg; TNF-α (Cetus Corp., Emeryville, CA), specific and activity 2.25×10^7 U/mg, IL-4 (Biosource International), specific activity, 2×10^6 U/mg. IL-2 (Cetus Corporation) specific activity 18×10^6 IU/mg, IL-12 of specific activity 5×10^6 U/mg was a kind gift from Dr Stanley Wolf, Department of Immunology, Genetics Institute, Cambridge, MA.

Synthetic peptides. The HER-2 peptides used were: E75 (369-377) GP2/F53: (IISAVVGIL, 654-662), and F57 (IHLNGSAYSL, 439-447). GP2 and F57 define HER-2 CTL

epitopes distinct from E75 (8-9). The modified Muc-1 peptides used were D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The FBP peptides used were: E39 (FBP,191-199, EIWTHSYKV), and E41 (FBP, 245-253 LLSLALML). The Amino Enhancer of Split (AES) peptide used was G76:GPLTPLPV. FBP and AES peptides were recently identified to be recognized by ovarian and breast CTL (10,11). The positive control CTL epitope used was the influenza matrix peptide (58-66): GILGFVFTL, designated as M1. M1 forms an immunodominant epitope recognized by memory CTL in healthy donors (15). All peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center and purified by HPLC. Peptides were 95-97% pure by amino acid analysis. Peptides were dissolved in PBS and stored frozen at -20°C in aliquots of 2 mg/ml.

Immunofluorescence. Antigen expression by DC and T-cells was determined by FACS using a flow-cytometer (EPICS - Profile Analyzer, Coulter Co., Hialeh, FL). DC were defined by the presence of CD13 and absence of CD14 marker after culture in GM-CSF and IL-4. For phenotype analysis, DC were incubated with PE-conjugated anti CD13 mAb and FITC-conjugated mAb specific for a surface Ag. For determination of the effects of cytokines, peptides and T-cells on surface antigen expression, DC were incubated with the same amounts of cytokines and peptides as in T-cell activation assays for 24 h, and the levels of Ag expression were determined in the gated $CD13^+$ population.

Culture of PBMC-derived DC. $CD13^+$ DC were generated from freshly isolated PBMC following the established CD14 methods (17,18). Complete RPMI medium (containing 10% FCS) supplemented with 1,000 U/ml GM-CSF or 500 U/ml IL-4 was added to each well containing plastic-adherent cells and maintained for 7 days. T-cells were obtained from the plastic non-adherent PBMC by removal of $CD16^+$ and $CD56^+$ cells. $CD8^+$ cells were isolated by removing first the $CD4^+$, and then the $CD16^+$ and $CD56^+$ cells from the non-adherent population using Dynabeads (Dynal, Oslo, Norway). $CD8^+$ subpopulations were obtained using anti-CD45RO mAb and anti-CD45RA mAb (UCHL-1, Dako) as described (15). After depletion, the resulting cells were 97% $CD8^+$ as determined by flow cytometry.

T-cell stimulation by peptide pulsed DC. DC were plated at $1.2x10^5$ cell/well in 24-well culture plates, and pulsed with peptides at 50 μg/ml in serum-free medium for 4 h before addition of responders. TNF-α (50 U/ml) was added to DC for the last hour to stimulate Ag uptake and presentation (17). Autologous, isolated $CD8^+$ cells or isolated $CD8^+$ cells ($CD45RO^+$ and CD45RO + cells depleted) in RPMI 1640 containing 10% HS were added to DC at $1.5x10^6$/ml, followed by IL-12. IL-2 was added 12-16 h later to each well. For inhibition studies, mAb specific for B7.1, B7.2, HLA-A2 and isotype control MOPC myeloma were added to DC or tumor cells, 1 h before responders in amounts reported to be inhibitory by the manufacturers. Anti CTLA-4 and CD40L mAb were added to T-cells 1 h before they were added to cultures. The effects of peptides and cytokines on T-cell survival were determined by counting the numbers of recovered viable cells, and determining the numbers of $CD8^+$ and $CD4^+$ cells in the sample by flow cytometry. Specific proliferative responses to E75 were determined by measuring the incorporated radioactivity in equal cell numbers pulsed with 1 μCi of (^{3}H)-TdR (19).

CTL and cytokine assays. Recognition of peptides used as immunogens by CTL was performed as described (8). Equal numbers of viable effectors from each well were used in all assays. Supernatants collected at 6, 24, or 48 h were tested in duplicate for the presence of IL-2, IL-4, IL-10, IL-12 and IFN-γ using cytokine ELISA-kits (Biosource International, Camarillo, CA) or R&D systems as described (19) with a sensitivity of 4-7 pg/ml. IL-12 was detected using an ELISA kit which recognizes both p40 and the natural heterodimeric molecule.

Results

$CD8^+$ cells from healthy donors display specific IFN-γ secretion within 24 h of contact with HER-2 peptide E75 potentiation by IL-12. To address whether stimulation with E75 induce cytokine responses, plastic-non-adherent PBMC from healthy donors were stimulated with autologous DC pulsed with E75 (DC-E75) or as control with DC which were not pulsed with peptides (DC-NP). Supernatants were collected 20-24 h later and tested for the presence of IFN-γ. Since the frequency of E75-specific responders may be low, we rationalized that addition of low concentrations (100-300 pg/ml) of IL-12 will amplify the levels of IFN-γ induced by E75, thus increasing the likelihood of detection of E75-responsive T-cells. IL-12 acts as co-stimulator for IFN-γ induction from T-cells by Ag, but by itself is a weak inducer of IFN-γ in T-cells (20,21). Results in Fig. 1 show the pattern of IFN-γ responses to E75 from four healthy donors, in the absence or presence of IL-12. In donor 1 in some experiments performed over time E75 rapidly induced IFN-γ without exogenous IL-12 (Fig. 1A), while in donor 2 (Fig. 1B) IL-12 was required to induce detectable IFN-γ levels to E75.

Since these experiments were performed with populations and not with isolated $CD8^+$ cells, these experiments were repeated three times at weekly intervals with donor 3 to address whether rapid IFN-γ induction and its amplification by IL-12 was not an isolated event. From donor 3 it was possible to obtain repeatedly peripheral blood over six months. Most experiments shown here were performed with donor 3 and were confirmed with at least one randomly selected $HLA-A2^+$ donor. Each experiment shown in Fig. 1C was performed with blood samples collected in different days. We used each time freshly cultured DC, plastic non-adherent PBMC from the same sample, and since the amount of blood was small, variable concentrations of E75 (20-50 μg/ml) and IL-12 (150 or 300 pg/ml). The results of stimulation experiments over a period of 3 weeks confirm that the pattern of responses observed with donors 1 and 2 was not an isolated event. At these E75 concentrations, IFN-γ was undetectable unless its induction was amplified by IL-12. In donor 5 E75 in high concentrations (100 μg/ml) induced high levels of IFN-γ within 24 h in the absence of exogenous IL-12

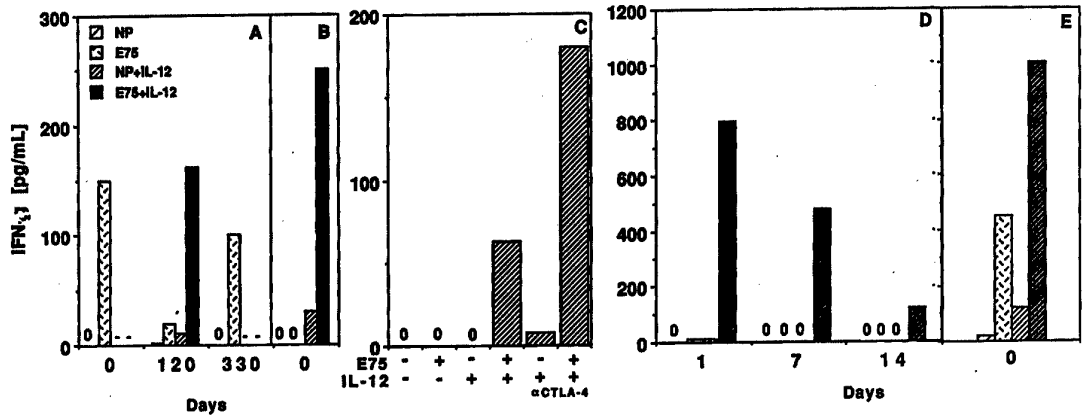


Figure 1. Freshly isolated, unstimulated plastic non-adherent PBMC from healthy donors (A, B, D and E) and breast cancer patients (C) specifically secrete IFN-γ within 20 h in response to E75. A, Donor 1. PBMC were collected on days 0, 120, and 330 counting as day 0 the date of first collection and stimulated with E75 at 50 μg/ml. (-) indicate not tested. B, Donor 2. E75 at 50 μg/ml. C, Unimmunized breast cancer patient. Supernatants were collected at 24 h after stimulation with 50 μg/ml E75. IL-12 was used at 3 U (330 pg/ml). D, Donor 3. Days 7 and 14 indicate the days after the first stimulation when the experiment was repeated with fresh PBMC. Day 1, 50 μg/ml E75 + 330 pg/ml IL-12; day 7, 25 μg/ml E75 + 150 pg/ml IL-12; day 14: 20 μg/ml E75 + 150 pg/ml IL-12. E, Donor 5, 100 μg/ml E75 + 330 pg/ml IL-12. Differences between the levels of IFN-γ induced by E75 + IL-12 and E75 alone or IL-12 alone were considered significant ($p<0.05$).

(Fig. 1C, column E). Similar results were obtained with donor 4 (Fig. 6A) which was tested in a separate experiment but not with donor 6 (Fig. 3B).

IL-4 was not detected in the E75 stimulation supernatants while the levels of IL-10 determined in the same experiment did not exceed 10 pg/ml during the first 96 h of E75-stimulation (data not shown). Similar results were obtained with 5 of 6 unimmmunized HLA-A2$^+$ breast cancer patients tested in the same conditions. Representative results with one patient in Fig. 1C also show that the IFN-γ response was also enhanced by α-CTLA4 mAb. These results suggest that there are significant numbers of E75-reactive T-cells in the PBMC of unimmunized healthy individuals and cancer patients that could be readily recalled following a primary stimulation *in vitro* by E75 ± IL-12.

T-cells from healthy donors secrete IFN-γ within 24 h in response to tumor cells in the presence of IL-12. Identification of E75 reactivity with high frequency in the PBMC raise the question of the potential of these cells for tumor recognition. To address this question, we investigated whether freshly isolated donor 1 T-cells recognized better the HLA-A2 matched tumor SKOV3.A2 compared with the non-matched SKOV3. SKOV3 and SKOV3.A2 are identical but the latter express a transfected HLA-A2 gene. Thus, donor 1 and SKOV3.A2 (HLA-A2, 3, 28, B18, 35) shared only HLA-A.2. We wanted to know whether the same or higher levels of IFN-γ will be induced in T-cells responding to Ag presented by allo-MHC-I compared with common HLA-A2. The results, Fig. 2A show that T-cells secreted low IFN-γ levels in response to SKOV3.A2. IFN-γ was not detected in response to SKOV3. In the presence of IL-12, IFN-γ was detected in both cultures within 24 h, but the levels were significantly higher in response to SKOV3.A2 than to SKOV3, suggesting that HLA-A2 restricted activated T-cells are present in this donor. To verify that IFN-γ production was the result of

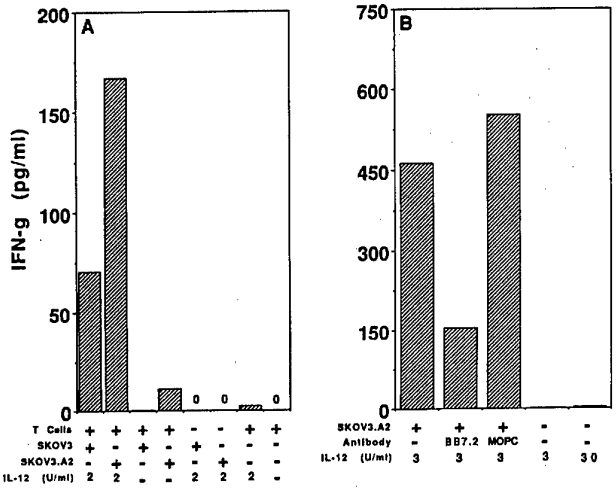


Figure 2. A, Freshly isolated T-cells from donor 1 produce higher levels of IFN-γ within 24 h of co-culture with SKOV3.A2 compared with SKOV3. ($p<0.05$, by the Student's t-test). B, IFN-γ response to SKOV3.A2 is dependent on HLA-A2 recognition. Experimental conditions as described in the Materials and methods.

HLA-A2 recognition, SKOV3.A2 cells were incubated either with BB7.2 mAb (α-HLA-A2) or with a non-specific isotype control Ab (MOPC). The results (Fig. 2B) confirmed that most of the IFN-γ was produced in response to HLA-A2, and was not increased by higher concentrations of IL-12. Thus, freshly isolated T-cells from healthy donors can recognize tumors in an MHC restricted fashion without previous *in vitro* stimulation, suggesting that activated effectors are present in these cells.

E75 induce IFN-γ secretion from activated memory cells. Requirement for cognate Ag. Rapid induction of IFN-γ by

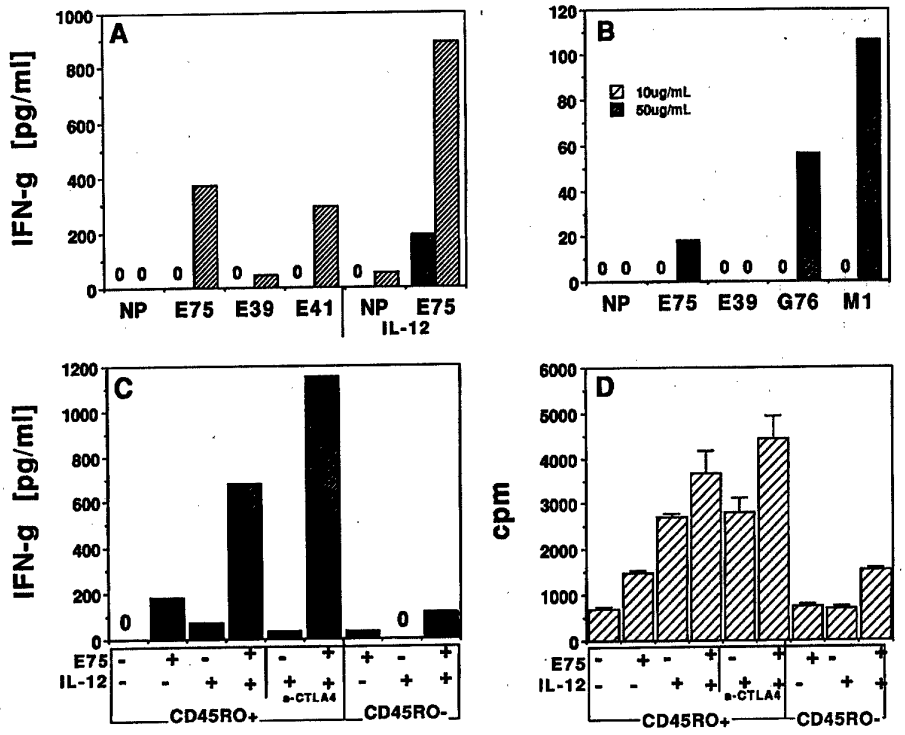


Figure 3. A, Induction of IFN-γ by E75 within 6 h (narrow hatching), and 48 h (wide hatching) from isolated CD8+ cells from donor 3. All peptides were used at 10 μg/ml; IL-12 at 330 pg/ml. B, IFN-γ induction from donor 6 by tumor peptides E75, E39, G76 and positive control M1 within 20 h in the absence of exogenous IL-12 is Ag and its concentration dependent. IFN-γ by G76 and M1 was significantly different from control NP cultures ($p<0.05$). C, IL-12 and αCTLA-4 mAb synergize in enhancing IFN-γ induction in response to E75 in isolated CD8+ CD45RO+ cells from donor 3. Equal numbers (10^6) of CD8+ CD45RO+ and CD8+ CD45RO cells were used as responders in each well. D, E75 in the presence of IL-12 and αCTLA-4 enhanced only marginally CD8+ cells proliferation. Equal numbers of CD8+ CD45RO+ and CD45RO+ depleted (CD45RO-) were collected from cultures after 48 h and used to determine differences in the rate of proliferation by E75. 10^5 donor 3 live cells were incubated with of ^{3}H-TdR for 8 h. Differences between IFN-γ levels in columns 2, 3, 4, 5, and 6 are significant ($p<0.05$). The experiment was performed in tetraplicate. Differences in ^{3}H-TdR are significant for all the (±) E75 groups by the Student's t-test, but the stimulation indexes (SI) are ≤2.0. Differences between groups IL-12-αCTLA4, and IL-12 alone are not significant, similarly differences between E75 + IL-12 and E75 + IL-12 + αCTLA-4 are not significant.

E75 raised the questions whether E75 and other tumor peptides prime naive T-cells or whether activated T-cells of this specificity are present in these healthy donors. Naive T-cells and resting memory cells require cell cycling (at least 1-2 divisions) i.e., minimum 30-40 h after Ag stimulation to secrete IFN-γ, while activated memory effector CTL respond to Ag by IFN-γ without requirements for additional cycling (i.e., O divisions) (13). Thus from memory effectors IFN-γ can be detected within 6-24 h (15).

To establish whether the rapid IFN-γ induction by E75 is a property of existent activated CD8+ cells and not the result, of initiation of activation of naive cells, isolated CD8+ cells from donor 3 were stimulated with DC-E75 in the presence or absence of IL-12. Supernatants were collected 6 h later and analyzed for IFN-γ. Rapid induction of IFN-γ within 6 h was observed only in cultures containing E75 + IL-12 (Fig. 3A). The levels of IFN-γ continued to increase over the next 48 h. At this time, IFN-γ was detectable even from the cultures that did not receive IL-12. The levels of IFN-γ were dependent on Ag sequence, since FBP peptides, E39 and E41, mapping CTL epitopes showed similar (E41) or weaker (E39) abilities than E75 to induce IFN-γ. Secretion of IFN-γ was Ag concentration dependent, indicating that E75 specific T-cells and not NK cells were the source of this cytokine (presented from a separate experiment in Fig. 5). Similar rapid IFN-γ induction by E75 + IL-12 within 12 h was observed with donors 1 and 4 (not shown) confirming that activated E75-reactive T-cells are present in healthy individuals.

To verify that the IFN-γ induction in tumor Ag-reactive CD8+ cells from PBMC is as rapid as the response of activated T-cells reactive with conventional Ag, the experiment was repeated in the absence of IL-12 with CD8+ cells from donor 6, using E75, E39, and the AES peptide G76 as stimulators. The dominant HLA-A2 restricted CTL epitope from influenza matrix (M1:58-66) was used as positive control. A rapid IFN-γ response to M1, is commonly used to define the presence of activated memory cells to influenza in healthy individuals (15). All peptides were used at the same concentration. The results in Fig. 3B show that in this donor, G76, and M1 at 50 μg/ml (~50 μM) induced IFN-γ within 20 h even without exogenous IL-12. At 10 μg/ml none of these Ag induced detectable IFN-γ. The magnitude of response was Ag dependent. M1 induced the highest levels of IFN-γ. The IFN-γ levels induced by G76 were significantly lower. The response to E75 was borderline. These results also show that the response is peptide-specific since E39 did not induce IFN-γ.

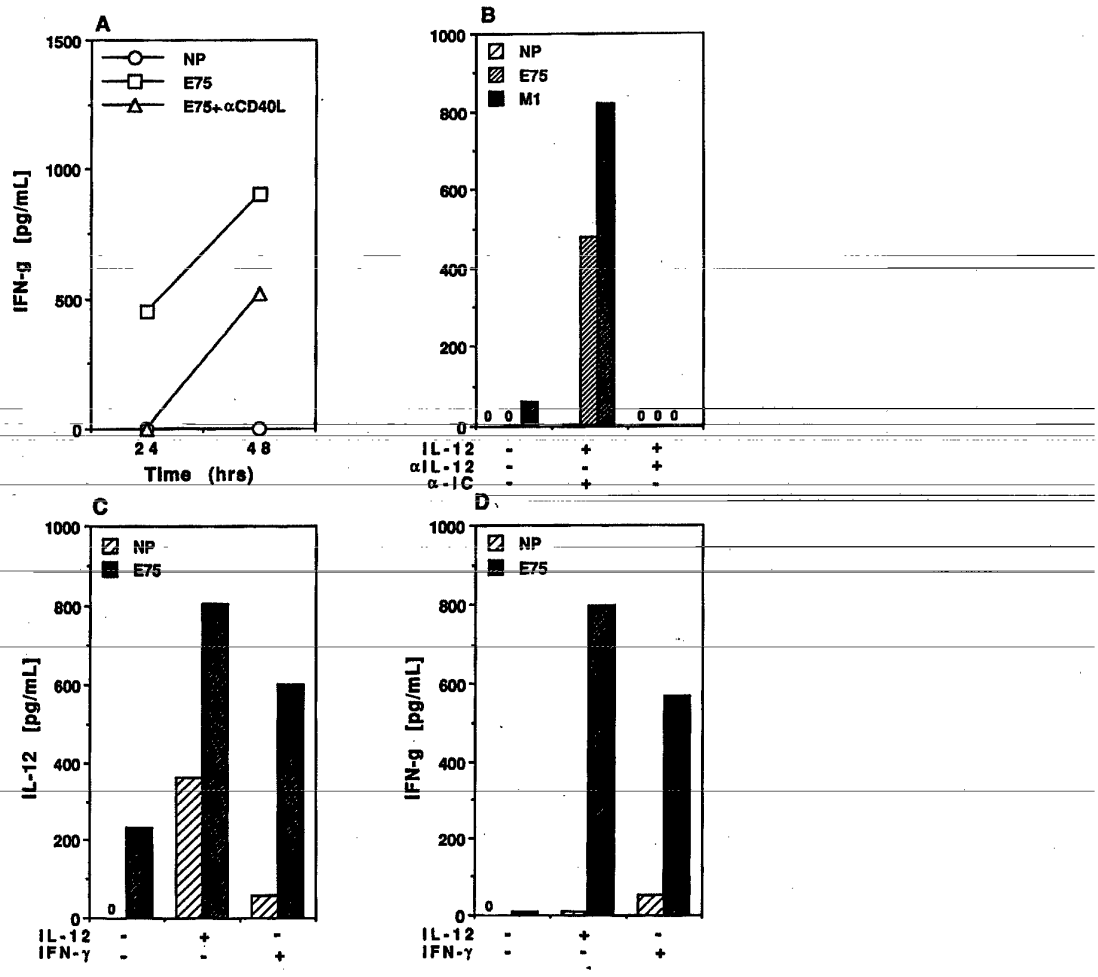


Figure 4. Induction of IFN-γ from E75-reactive CD8⁺ cells requires CD40L-CD40 interactions and is controlled by IL-12. A, Isolated CD8⁺ cells from donor 1 were stimulated with 100 μg/ml E75 in the presence of αCD40L mAb or isotype control mAb (NP) indicates background levels from cultures not stimulated with peptide (B). The same responders were stimulated with E75 and M1 at 25 μg/ml in the absence (-) or presence (+) of IL-12 (330 pg/ml). α-IC indicate isotype control Ab. The levels of IFN-γ induced by M1 were significantly higher than the levels induced by E75 in the presence or absence IL-12 ($p<0.05$). C and D, In a separate experiment responders were stimulated with E75 at 25 μg/ml in the absence (-) or presence (+) of IL-12 (330 pg/ml) or IFN-γ (50 pg/ml). NP indicate no peptide.

To establish that IFN-γ is induced in response to cognate Ag and confirm that the differences between the levels of IFN-γ are dependent on the Ag, the experiment was repeated with donor 5, using three HER-2 peptides (E75, GP2 and F57) reported to be recognized by CTL-TAL (10-12) and as control the unnatural Muc-1 peptide D132. D132 was obtained by replacing Pro (P2, P4) with a P2 anchor (L) to ensure HLA-A2 binding and a charged residue in P4 (D) to perturb a TCR contact in the corresponding Muc-1 sequence. The HLA-A2 stabilizing ability of these peptides decreased in the order F57>D132>E75>GP2. The IFN-γ levels in the presence of IL-12 were: D132:105 (unnatural peptide), F57:380, GP2:740, and E75:980 pg/ml, respectively. These results were confirmed with donor 1: NP, 20 pg; D132, 25 pg; E75, 160 pg. The rapid IFN-γ response was 6-9-fold higher for E75, previously reported to be recognized by TIL/TAL, than for the unnatural peptide D132 which is not present in the donor. Since these differences were observed at the same Ag concentration, they likely reflect differences in peptide stimulatory potency and/or frequency of existent Ag-specific activated responders.

To establish that the IFN-γ response to tumor Ag originated from effector or memory cells, but not from naive cells, CD8⁺ CD45RO⁺ and CD8⁺ CD45RO⁻ cells were isolated from the same blood sample from donor 3. Both memory and effector cells express the CD45RO antigen. Equal numbers of each population were tested in parallel for IFN-γ induction and proliferation in response to E75 ± IL-12 (Fig. 3C and D). The results show that CD8⁺ CD45RO⁺ cells were the main producers of IFN-γ in response to E75 + IL-12. The levels of IFN-γ were by 5-fold lower when CD8⁺ CD45RO⁻ cells were used as responders. Comparison of the IFN-γ levels with the proliferative response demonstrated that E75 is a weak inducer of proliferation in both CD45RO⁺ and CD45RO⁻ cells. IL-12 did not synergize with E75 in increasing CD8⁺ cells proliferation. Although the overall levels of ^{3}H-TdR incorporation were higher in the presence than in the absence of IL-12, the stimulation indexes for E75 + IL-12-stimulated

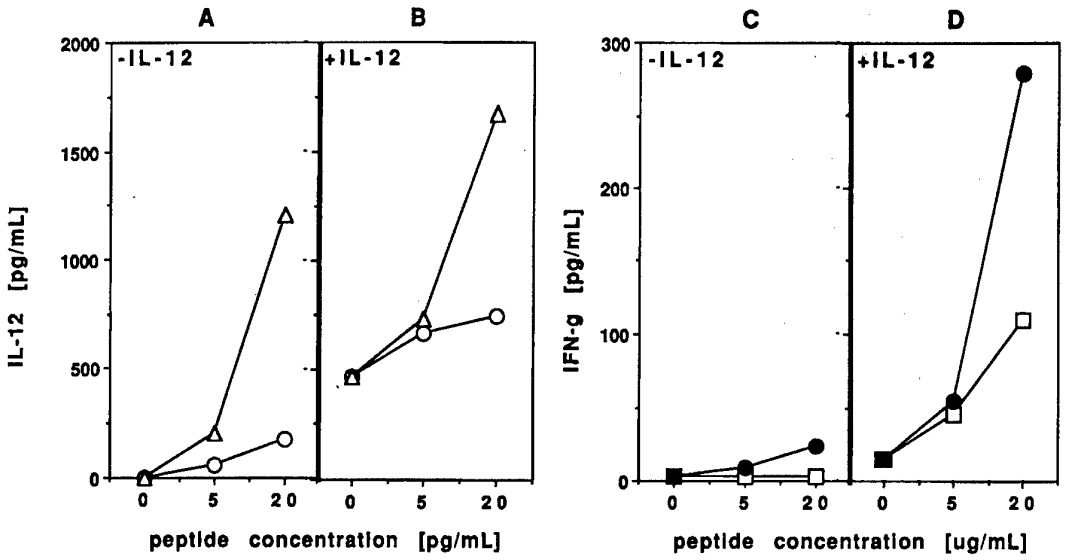


Figure 5. Concentration-dependent induction of IL-12 (A and B) and IFN-γ (C and D) by E75 and M1. ±IL-12 indicate that exogenous IL-12 was not added (-) or used (+) at 330 pg/ml. IL-12 and IFN-γ were determined from the same experiment from the same supernatants collected 20 h after stimulation. (○, ●), E75; (△, ▲), M1.

cultures were <2.0. In the presence of IL-12, blocking of CTLA-4 in responders increased the IFN-γ response to E75 compared with E75 + IL-12 by two fold. In the same experiment, the $CD8^+$ $CD45RO^+$ cells proliferation to E75 + IL-12 + αCTLA4 was not significantly different from E75 + IL-12.

E75-dependent induction of IL-12 from DC. Synergy with IFN-γ. To elucidate the IFN-γ induction pathways activated by E75 we first determined whether IFN-γ induction required CD40L-CD40 interactions between APC and T-cells. Isolated $CD8^+$ cells from donor 3 were stimulated with 100 μg/ml E75 in the absence of IL-12. Usually at this Ag concentration IFN-γ could be detected in the absence of exogenous IL-12 (Fig. 1C). αCD40L and isotype control Ab were added to cultures stimulated in parallel, and the IFN-γ levels were determined at 24 and 48 h. The results (Fig. 4A) show that IFN-γ secretion was significantly inhibited in the presence of αCD40L suggesting that IFN-γ induction required CD40L-CD40 interactions between activated T-cells and APC.

Since the CD40-CD40L interaction is the major pathway for T-cell dependent IL-12 induction from APC, this raised the question whether IFN-γ induction is controlled through IL-12 induced from APC. To address this question we determined the effects of neutralizing IL-12 on IFN-γ induction. Parallel cultures were stimulated with E75 or M1 in the presence of a neutralizing α-IL-12 mAb, and an isotype control mAb (IC). E75 and M1 were used at 25 μg/ml. At this concentration E75 but not M1 required exogenous IL-12 to detect induced IFN-γ. The results in Fig. 4B show that IFN-γ production in response to both E75 + IL-12 and M1 + IL-12 was completely inhibited by α-IL-12, suggesting that induction of IFN-γ is dependent on IL-12.

To address whether E75-reactive $CD8^+$ cells induced IL-12 from DC, we determined the levels of IL-12 in the same experiment in response to E75 and control (no peptide). In addition, we tested in parallel whether IL-12 and IFN-γ are cofactors for IL-12 induction by E75. E75 rapidly induced IL-12 (Fig. 4C). Exogenous IL-12 had a modest synergistic effect with endogenous IL-12 in determining the overall IL-12 levels in the culture (240 pg endogenous + 360 pg exogenous = 600 pg, compared with 780 pg total detected). This suggested that the co-stimulatory effect of exogenous IL-12 is not due to its own amplification. In contrast, IFN-γ at 50 pg/ml (the level induced by M1 in the absence of IL-12 in Fig. 4B) synergized with E75 in enhancing IL-12 levels: 240 + 50 = 290 pg vs. 600 pg/ml total detected (Fig. 4C, column 3). Higher levels of IFN-γ were also detected in the wells stimulated with E75 + 50 pg IFN-γ, but not in the well stimulated with IFN-γ alone, demonstrating that IFN-γ can amplify its own response only in the presence of the tumor Ag (Fig. 4D). This effect did not require exogenous IL-12 because the levels of IL-12 induced by E75 + IFN-γ (shown in Fig. 4C) were apparently above the threshold needed to costimulate IFN-γ induction.

These results suggested that E75-reactive $CD8^+$ cells induced IL-12, when recognized E75 on DC. Since IFN-γ was not detected at 25 μg E75 in this experiment, this suggested that compared with M1 the induced IL-12 levels were insufficient to co-stimulate IFN-γ. To address this question we determined in parallel the levels of IL-12 and IFN-γ induced in a concentration-dependent fashion by E75. The experiment was performed in the absence or presence of exogenous IL-12. We used M1 as a positive control. IL-12 production was Ag concentration dependent for both peptides (Fig. 5A). The levels of IL-12 induced by M1 were significantly higher than the levels induced by E75. Exogenous IL-12 did not change the dose-response pattern of IL-12 induced by the either Ag suggesting that within 24 h it did not induce higher levels of IL-12 by itself. Again M1 induced low levels of IFN-γ in the absence of exogenous IL-12.

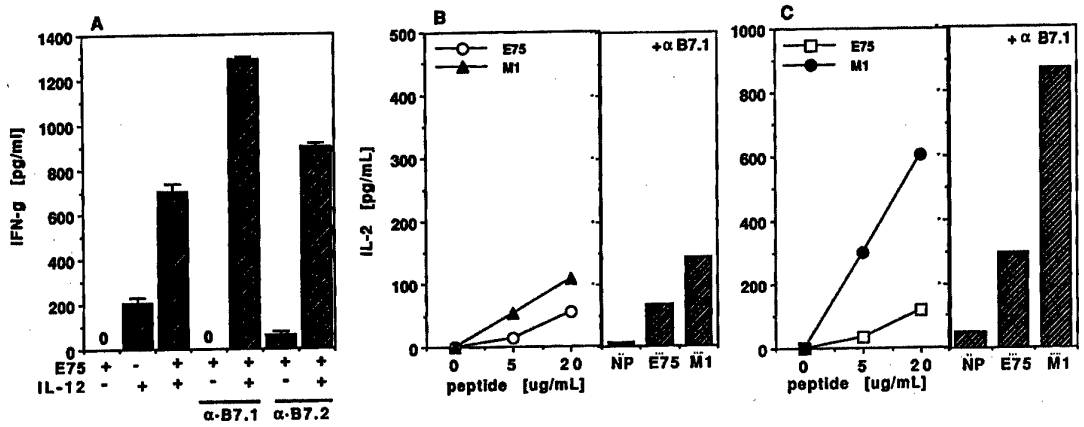


Figure 6. A, The synergy between E75 and B7.1/B7.2 mAb in enhancing IFN-γ requires the presence of IL-12. In the absence of IL-12, low levels of E75 induced IFN-γ were detected in the presence of αB7.1 at 48 h (50 pg/ml). B and C, E75 synergize with αB7.1 in enhancing IL-2 induction. Exogenous IL-2 was added at 200 pg/ml, 8 h before supernatant collection. C, Results indicate pg/ml IL-2 after subtracting the levels of IL-2 resolved in the control well (179 pg). Both peptides were tested in the same experiment performed in parallel. IL-2 was determined in the same experiment. A, donor 4 (B and C) donors 3 isolated CD8⁺ cells. αB7.1 and B7.2 mAb were added to the DC 1 h before addition of responders (hatched) indicates levels of IL-2 determined at 24 h from cultures stimulated with either peptide at 20 μg/ml in the presence of αB7.1.

The increase in overall levels of IL-12 following exogenous IL-12 addition was paralleled by increase in levels of IFN-γ in response to both Ag. Thus the role of exogenous IL-12 is to compensate for the insufficient levels of IL-12 secreted by DC at encounter with tumor Ag reactive CD8⁺ cells (Fig. 5A vs. B). The levels of IL-12 induced by 5 μg M1 were similar with the levels of IL-12 induced by 20 μg E75. At these peptide concentrations IFN-γ was not detected in response to E75. Therefore, the results indicate that there is a minimum required level of endogenous IL-12 to be present in cultures for IFN-γ to be detected in response to tumor Ag. When IL-12 is below this level, IFN-γ cannot be detected in response to peptide stimulation (Fig. 5A and C). This deficiency was compensated sometimes by high concentrations (>100 μg/ml) of E75, (Figs. 1E and 4A), which induced substantially higher levels of IL-12 (>1,600 pg/ml). (not shown).

Primary stimulation with E75 induced IL-2 in healthy donor CD8⁺ cells. Enhancement by αB7.1. Regulation of T-cell response by Ag involves at least two major mechanisms: the first by direct induction of IL-12 from APC, through CD40L-CD40 and the second through the B7-CD28 interaction (22,23). The former apparently controls the IFN-γ induction, while the latter controls the IL-2 secretion and responsiveness to IL-2 through high affinity IL-2R induction (24,25). The first pathway can also positively impact on the second through up-regulation, among others, of costimulatory molecules of the B7 family. A B7-CD28 dependent costimulatory pathway can also mediate a functional type 1 cytokine response (26,27) and synergized with IL-12 (28). Results in Figs. 1C and 3C show that α-CTLA-4 enhanced IFN-γ induction in response to E75 + IL-12. This suggested that the responders may be activated but negative signaling after ligation of B7 reduces the response. If this is the case, blocking of B7 is expected to reverse the inhibitory effects. This will be evidenced by enhanced induction of IFN-γ, IL-2 and proliferation. To directly address the role of B7 in E75-induced cytokines, we investigated the role of B7-1 in IFN-γ and IL-2 induction.

To verify that induction of IFN-γ by E75 is enhanced by B7 blocking, the experiment was repeated with donor 4 using isolated CD8⁺ cells. Since B7.1 and B7.2 were expressed on DC at different levels, we used alternatively αB7.1 and αB7.2 mAb to block the receptor ligation. In the absence of IL-12, αB7.1 did not enhance IFN-γ in response to E75, while αB7.2 co-stimulated IFN-γ induction only weakly (=50 pg/ml). However, in the presence of IL-12, αB7.1 enhanced the E75 + IL-12 induced response by two fold. The potentiating effect of B7.2 was much weaker. The results (Fig. 6A) confirmed that the synergy between E75 and αB7 for high IFN-γ secretion within the first 24 h required IL-12. Since blocking of B7-CTLA4 synergized with Ag + IL-12 in IFN-γ production these results confirmed that the responders were activated CD8⁺ cells.

To characterize the ability of E75-reactive CD8⁺ cells to produce IL-2 the experiment was repeated with donor 3 using M1 as positive control. Since in activated T-cells blocking of B7/CTLA-4 was reported to reverse the state of tolerance of T-cells for proliferation through induction of IL-2 (29), we investigated whether in our system B7.1 was required for IL-2 induction in response to E75 and M1. E75 and M1 were used at the same concentrations (5 and 20 μg/ml) as for IL-12 and IFN-γ induction in the experiment shown in Fig. 5. E75 induced IL-2 in a concentration-dependent manner in the absence of αB7.1. The IL-2 levels at 5 and 20 μg/ml E75 were 4 and 2-fold lower, respectively, than the levels induced by M1. Twenty μg E75 induced the same levels of IL-2 as 5 μg M1. αB7.1 did not inhibit IL-2 production, but in contrast it had a slight enhancing effect by 20% (E75) and 40% (M1) compared with peptide alone. Even in the presence of αB7.1 the levels of IL-2 induced by M1 were at least two fold higher than the levels induced by E75 (Fig. 6B). Thus previously *in vivo* activated CD8⁺ cells in this healthy donor are not tolerized/ anergic with respect to IL-2 production.

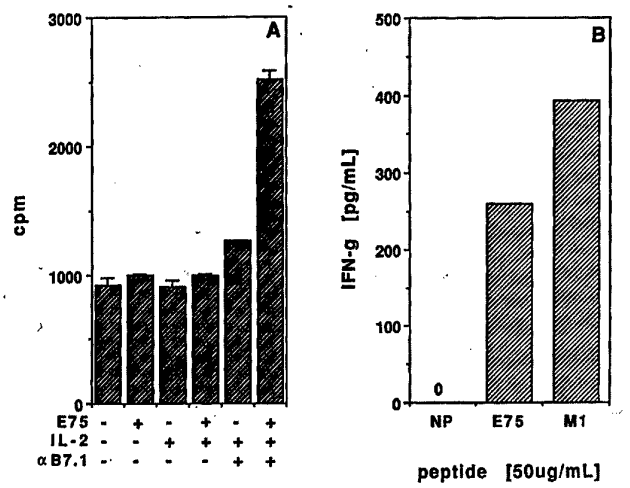


Figure 7. A, Proliferative responses of CD8+ populations. αB7.1 enhanced the rate of proliferation induced by priming with E75 in the presence of IL-2. CD8+ cells from donor 3 were stimulated with DC-E75 for 72 h. 10^5 cells were collected from each well, and pulsed with ^{3}H-TDR for the last 8 h. All measurements were performed in tetraplicate. Results indicate cpm/50,000 cells ± SD. B, IFN-γ response of preactivated donor 3 CD8+ cells. CD8+ cells primed with DC-E75 + IL-12 and maintained in culture for a total of 5 days were rested for 24 h in the absence of IL-2 and restimulated on day 6 with DC-E75 without exogenous IL-12. Supernatants were collected at 20 h. Results indicate pg/ml/10^6 cells.

Since IL-2 acts as a survival factor, exogenous IL-2 at 200 pg/ml was added for the last 8 h before supernatant collection in a parallel experiment. The results in Fig. 6C show the IL-2 levels induced by E75α, M1 ± αB7.1, after correction by subtracting the amount of exogenous IL-2 recovered in the control wells. The levels of IL-2 induced by E75, increased by two fold compared with the levels of IL-2 induced by E75 in IL-2 absence. In the same conditions the levels of IL-2 induced by M1 increased by 5-fold. αB7.1 increased significantly, by 2.5-fold, the levels of IL-2 induced by E75 but less, by 1.5 the levels of IL-2 induced by M1 without αB7.1. Again, levels of IL-2 induced by 20 μg E75 + αB7.1 were similar with the levels induced by 5 μg M1. The IL-2 response in E75-stimulated cells was stable for the next 24 h. At 48 h, the IL-2 levels for 5 and 20 μg E75 were 88 and 120 pg/ml respectively. Thus, when *in vivo* E75-activated cells remain viable they are able to produce more IL-2 in response to blocking of B7.1 confirming that they are not anergic.

Priming with E75 + B7.1 + IL-2 weakly increased CD8+ cells proliferation (Fig. 7A) similarly with E75 + CTLA-4 (Fig. 3D). This suggested that these cells undergo only limited expansion in response to E75. E75 + IL-2 + αB7.1 induced weak proliferation at 72 h, stimulation index (SI) = 2.53. IL-2 + αB7.1 without E75 induced borderline proliferation (SI = 1.28) compared with control E75-stimulated cultures: (SI = 1.0, i.e., 993 cpm ± 50). Thus, in the presence of IL-2 with blocking of B7.1, CD8+ cells responded to the E75 signal better than in the absence of αB7.1. Thus the negative signaling through B7-CTLA4 could provide a mechanism by which the proliferation of these cells is inhibited.

To address the possibility that the cells were not becoming non-responsive as a result of Ag activation, and required IL-12 as a consequence of cytokine-mediated tolerization, E75 + IL-12 and M1 + IL-12 primed cells from donor 3 were restimulated with E75 and M1 respectively in the absence of IL-12. IFN-γ was determined 20 h later. The results in Fig. 7B show that on a per cell basis these cells responded to Ag stimulation, in the absence of IL-12, with even higher levels of IFN-γ. Thus CD8+ cells present in healthy donors, after *in vitro* stimulation became more responsive to restimulation through the TCR, regarding proliferation and IFN-γ production, suggesting that under these stimulation conditions the Ag is not tolerogenic.

Primary stimulation with DC - E75 ± IL-12, αB7/CTLA-4 induce Ag-specific.cytolytic activity infrequently. Ag-specific CTL activity was observed at priming only in two donors and inconsistently over time in two other donors. This suggested that stimulation of this function required different activation thresholds, which could not be reached by DC-E75. Ag-specific CTL activity was detected in two other donors after 3-4 stimulations suggesting that differences in frequency together with the slow rate of division of these cells did not allow CTL effectors to reach the critical numbers for specific lysis to become evident (Anderson *et al*, preliminary data). These results show that activated tumor Ag reactive CD8+ cells are present in healthy individuals and are not tolerized. They are responsive to stimulation through the TCR since they rapidly secrete IL-2, IFN-γ and IP-10 (Lee *et al*, preliminary data) as well as induce IL-12 in DC. Tumor Ag such as E75, are not tolerogenic but weak inducers of IL-2 and IL-12 and even weaker inducers of proliferation, suggesting that they are weak/partial agonists for activation of effector functions.

Discussion

In this study, we investigated the presence of activated, tumor Ag- and tumor-reactive cells in CD8+ cells freshly isolated from PBMC of healthy donors. The IFN-γ, IL-2, and IL-12 induced by E75 and E75-reactive CD8+ cells mirrored the response to M1 conventially used to define the presence of activated CD8+ influenza specific cells in healthy individuals. We found that E75-reactive CD8+ cells are present in some donors in a state wherein they can secrete IFN-γ within 6-12 h of antigen exposure. Our results show that T-cells reactive with HER-2, FBP and AES peptides exist in healthy donors and they are rapidly activated by tumor Ag in a similar fashion with the memory cells reactive with viral Ag. The fact that in the same donor Ag induced IFN-γ was detectable at different time points in the absence or presence of IL-12 may reflect changes in the numbers of E75-specific cells or in their state of activation. Since the IFN-γ response was obtained primarily from CD45RO+ cells, and was amplified by α-CTLA4/αB7 only in the presence of IL-12, while the IL-2 response was amplified by αB7.1, our results suggest that E75-reactive activated CD8+ cells are frequently present in healthy individuals.

In most donors, high IFN-γ induction by Ag was detected within 20 h when costimulated by IL-12. The exogenous IL-12 requirement for detection of IFN-γ was dependent on the Ag

sequence and concentration. The sequence dependency for peptides derived from the same protein was supported by the fact that in the same donor the IFN-γ responses to other HER-2 peptides of higher (F57) or weaker (GP2/F53) binding affinity to HLA-A2 were significantly lower than to E75. This also raised the possibility that $CD8^+$ cells of different affinities for each tumor Ag and at different frequencies may be present in the same individual. This was suggested by the facts that: i) IFN-γ responses to FBP peptide E41 and to the AES peptide G76 both of lower HLA-A2 affinity than E75 showed similar or higher IFN-γ levels with responses to E75 and ii) at high (100-150 μg/ml) E75 concentration exogenous IL-12 was not required for IFN-γ induction. IL-12 was required when E75 was used at 5-20 μg/ml. Similarly, influenza-matrix, M1-reactive-$CD8^+$ cells from the same donor required exogenous IL-12 when stimulated with 5 μg but not with 20 μg of M1. For both Ag (E75 and M1) the requirement for exogenous IL-12 for IFN-γ induction inversely correlated with the amount of endogenous IL-12 induced.

The presence of activated $CD8^+$ cells specific for E75 in the PBMC of healthy donors is also supported by the fact that these cells induced rapidly IL-12 from APC. IL-12 production increased in direct proportion to E75 concentration. Since induction of IL-12 require CD40 triggering, only when T-cells are involved (24), these results support the possibility that the responders to E75 and the other tumor Ag are activated $CD8^+$ cells. We found that the amount of IL-12 induced in APC by peptide-reactive $CD8^+$ cells should be above a certain level for IFN-γ to be detected in response to E75/M1. In the donors studied E75, at concentrations as high as 20 μM cannot induce sufficiently high levels of IL-12 required for mediation of IFN-γ costimulatory activity. The IL-12 dependent control of IFN-γ response to Ag may provide a mechanism for maintaining these cells in a non-responsive state. This may reflect the requirement for higher levels of signaling by E75 and the other tumor Ag (AES, FBP) for TCR-mediated activation of the existent reactive $CD8^+$ cells compared with conventional Ag (30). By comparing the levels of IL-12 and IL-2 induced by E75 and M1 at two Ag concentrations it appeared that M1 is at least 4-fold more potent than E75 in cytokine induction. Thus the lack of IFN-γ at low E75 concentrations did not reflect poor Ag presenting/T-cell-activating ability by DC used as APC.

To gain insight into the requirements for activation of these cells we investigated the mechanisms of activation that may be affected by E75 recognition. IL-12 production is amplified either by increased CD40L expression on T-cells or by endogenous IFN-γ production. Both pathways are dependent on Ag concentration (24). Although E75 stimulation increased CD40L levels on T-cells it did not increase the numbers of $CD40L^+$ cells. Using two-color FACS analysis (CD40L-PE vs. CD8-FITC) we found that at 20 h E75 stimulation increased the vertical (Y) mean for $CD40L^+$ $CD8^+$ cells in the upper right quadrant by three fold (from 13.3 to 34.8). Addition of IL-12 doubled the Y mean level to 70.9, but not the % positive cells. The $CD8^+$ $CD40L^+$ cells were in the range 0.2-0.3%. We also determined the expression of IL-2Rα (CD25), an indicator of responsiveness through TCR, on E75-stimulated cells. Stimulation with E75 + IL-2 increased only weakly the Y mean for $CD8^+$ IL-$2R\alpha^+$ cells, from 23.2 in control (no peptide + IL-2) to 27.2 in E75 + IL-2. Thus E75 appeared to have distinct potencies for induction of CD40L and IL-2Rα.

We demonstrated that IL-12 induction by E75 was not dependent on the presence of endogenous IFN-γ (Fig. 5). However, low (50 pg) of exogenous IFN-γ amplified the IL-12 response to E75. Therefore, low levels of IFN-γ induced by pathogens, or crossreactive Ag in the vicinity of these cells, *in vivo* may activate IFN-γ production by tumor Ag in these cells through a positive feedback loop: Ag1 + low IFN-γ→IL-12→IL-12 + Ag2→more IFN-γ. The exogenous IL-12 requirement for IFN-γ activation may be due to the fact that E75 is a weak inducer of CD40L. Low levels of CD40L in E75-reactive memory cells cannot induce the minimal levels of IL-12 to co-stimulate for IFN-γ production in response to E75. Another possibility which was not yet investigated but deserve attention in further studies is that the signal transduced by E75 in T-cells is also a weak inducer of the IL-12Rß chain, which is required for the high affinity IL-12Rß expression. The high affinity IL-12R increase the sensitivity of responders to lower concentrations of IL-12, and it is stabilized by IFN-γ (31).

Given our data demonstrating a role for CD40L and IL-12 in regulating IFN-γ induction from E75-reactive $CD8^+$ cells and the reports that IFN-γ induction and IL-12 responsiveness in T-cells can be also enhanced via CD28 we studied the involvement of B7-costimulation in IFN-γ and IL-2 production. The regulation of IFN-γ induction by IL-12 was confirmed by IFN-γ enhancement by mAb to B7/CTLA-4 only in the presence of IL-12.

An enhancing role for blocking of B7 was observed in IL-2 induction by E75. E75 alone induced IL-2 within 24 h, although at low levels. α-B7.1 increased E75-induced IL-2. The fold increase was higher for E75 than for M1 in the presence of IL-2. This suggested that E75-reactive $CD8^+$ cells do not differ from positive control, *in vivo* activated matrix-reactive $CD8^+$ cells, in their ability to induce/secrete: IL-12, IL-2 and IFN-γ. They differed in the overall amounts of cytokines secreted. Since 4-fold more E75 was required to induce the same levels of IL-12, IFN-γ, and IL-2, induced by M1, this raises the possibility that the activated $CD8^+$ cells are in a hyporesponsive state. Since these results were obtained only with few donors, additional studies using separated populations are required to address this point. The 2-4-fold difference in cytokine levels may not exclude the possibility that the frequency of E75-specific CTL is lower than the frequency of M1-specific CTL, or that within the E75-responders there are subpopulations endowed with high IFN-γ secretion activity.

Priming with E75 in the presence of IL-2, αCTLA-4 or αB7.1 although enhanced IL-2 production increased only marginally the responders proliferation. The reasons for this selective responsiveness are unknown. E75 + IL-12 primed cells secreted high levels of IFN-γ at restimulation with E75. Thus they maintained responsiveness through the TCR. However, their poor proliferative ability was not reversed by preculture in IL-2 as was expected if they were partially tolerized/anergic (Lee *et al*, preliminary data).

One possibility to be considered is that this functional dichotomy reflects a weak agonistic activity of E75 in that

the signal transduced through TCR can activate the IFN-γ, IL-12, IL-2 (this paper) and IP-10 induction (Lee *et al,* preliminary data) but sustains a slow division of these cells. The outcome of this slow division is that the frequency of specific cytolytic effectors increase slowly with each Ag stimulation. Based on $CD8^+$ $CD40L^+/IL\text{-}2R\alpha^+$ data the frequency of these cells is less than 10-3 but not less than 1/10,000-1/20,000. If a minimum frequency of 10^{-1}-10^{-2} is required for detection of specific CTL activity (at E:T = 10-20/1) (15), and these cells increase in number by 4-5-fold after 2 stimulations, to reach the minimum threshold of 10-fold increase will require more than 3 stimulations. This is in agreement with reports on restimulation requirements for induction of tumor cytolysis (32). This is also supported by the slow increase in lytic units specific for melanoma Ag in healthy donors compared with cancer patients (4). In support of our conclusions, it has been recently shown that: i) acquisition of cytotoxic function by activated CTL require at least one cell division (33) and ii) T-cells that survive as memory cells proliferate weakly during the expansion phase of an immune response (34).

Our work raised the intriguing possibility that activated HER-2 Ag-specific $CD8^+$ cells exist *in vivo* in healthy individuals. This would make sense since it is unclear how a peripheral repertoire of naive T-cells specific for tumor Ag could be maintained in healthy donors in the absence of Ag stimulation, and how it can survive only on cytokines secreted in *trans* by other cells. Their hyporesponsive state may be due to the increased threshold for self-reactivity as recently described in IEL from self-Ag^+ mice (35). This hyporesponsitivity may reflect the weak immunogenicity of the tumor Ag in the periphery for positively selected self-reactive cells (reviewed in ref. 36). Epithelial tissues expressing HER-2 or FBP may provide the epitope precursors and/or crossreactive immunogens due to physiological turnover, while IL-12 induced by pathogens at the same time may costimulate in *trans* IFN-γ secretion. Similarly, IL-2 from pathogen stimulated $CD4^+$ cells can drive their expansion or act as survival factor. Activated $CD8^+$ cells may not need professional APC for activation, since the B7-CTLA4 interaction may be in fact inhibitory. These populations may be maintained in steady-state by death of activated cells (AICD) expressing higher-affinity receptors for Ag. The affinity for the tumor Ag of the surviving population may decline over time. The surviving cells may be reactivated as CTL-TAL only when the tumor expresses very high concentrations of Ag (e.g., HER-2, gp100, FBP). At that time, tumor-derived IL-10, TGF, angiogenic chemokines, will inhibit the functional IL-12, IFN-γ and IP-10 response.

Identification of activated tumor-reactive memory $CD8^+$ cells in healthy individuals with high frequency raise several novel and possibly important implications for tumor immunosurveillance, and vaccine design: i) since such cells are present in healthy donors their rapid mobilization to mediate cytokine associated effector functions may be useful for maintaining a mechanism of control of tumor emergence in individuals at high risk or in patients with no evidence of disease. This may be achieved by periodic stimulation with low concentrations of tumor Ag plus IL-12. This should be particularly relevant for ovarian cancer where the recurrence rate is high. ii) The sensitivity of IL-12 induction and of the IL-12R to negative regulation by IL-10, may require, in cancer patients, approaches to neutralize regulatory cytokines at vaccination with tumor Ag if the activation of IFN-γ^+ effectors is aimed. ii) Naive $CD8^+$ cells primed by tumor Ag plus B7 costimulation, over time convert to memory cells. After death, due to repeated encounters with Ag, *in vivo*, the surviving cells of lower affinity for Ag will require even higher Ag concentrations for activation than at priming. Blocking of B7-CTLA4 will be unable to reverse negative regulation of CTL expansion if the tumor Ag cannot reach the threshold for TCR signaling for cell cycling. Thus, the weak signaling demonstrated by the wild-type tumor Ag would require development of different TCR agonists than the ones currently used, for activation of proliferation and rescue of the high affinity memory cells, to be used for vaccination. Therefore, the results reported above may be useful to develop approaches to activate cellular immunity to tumors.

Acknowledgements

The contribution of T.V. Lee and B.W. Anderson is equivalent, and they both should be considered as first authors. We thank Mr. Louis J. Soule for outstanding editorial assistance, and the Blood Bank personnel for donating blood repeatedly for this project. This work was supported in part by grant DAMD17-97-1-7098. Peptide synthesis was supported in part by the core grant CA 16672.

References

1. Houghton AN: Cancer antigens: immune recognition of self and altered self. J Exp Med 180: 1-4, 1994.
2. Pittet M, Valmori D, Dunbar PR, Speiser DE, Lienard D, Lejeune F, Fleischhauer K, Cerundolo V, Cerottini J-C and Romero P: High frequencies of naive melan-A/MART-1-specific $CD8^+$ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2-individuals. J Exp Med 190: 705-715, 1999.
3. Anichini A, Molla A, Mortarini R, Tragni G, Bersani I, DiNicola M, Gianni AM, Pilotti S, Dunbar R, Cerundolo V and Parmiani G: An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. J Exp Med 190: 651-667, 1999.
4. Marincola FM, Rivoltini L, Salgaller ML, Player M and Rosenberg SA: Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence of *in vivo* priming by tumor cells. J Immunother 19: 266-277, 1996.
5. Goldrath AW and Bevan MJ: Selecting and maintaining a diverse T-cell repertoire. Nature 402: 255-262, 1999.
6. Fisk B, Blevins TL, Wharton JT and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 181: 2109-2117, 1995.
7. Brossart P, Stuhler G, Flad T, Stevanovic S, Rammensee HG, Kanz L and Brugger W: Her-2/neu-derived peptides are tumor-associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by *in vitro* induced specific cytotoxic T lymphocytes. Cancer Res 58: 732-736, 1998.
8. Rongcun Y, Salazar-Onfray F, Charo J, Malmberg KJ, Evrin K, Maes H, Kono K, Hising C, Petersson M, Larsson O, Lan L, Appella E, Sette A, Celis E and Kiessling R: Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. J Immunol 163: 1037-1044, 1999.

9. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I and Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci USA 92: 432-436, 1995.
10. Peoples GE, Anderson BW, Fisk B, Kudelka AP, Wharton JT and Ioannides CG: Ovarian cancer-associated lymphocyte recognition of folate binding protein peptides. Ann Surg Oncol 5: 743-750, 1998.
11. Babcock B, Anderson BW, Papayannopoulos I, Castilleja A, Murray JL, Stifani S, Kudelka AP, Wharton JT and Ioannides CG: Ovarian and breast cytotoxic T lymphocytes can recognize peptides from the amino-enhancer of split protein of the Notch complex. Mol Immunol 35: 1121-1133, 1998.
12. Miller C, Ragheb JA and Schwartz RH: Anergy and cytokine-mediated suppression as distinct superantigen-induced tolerance mechanisms *in vivo*. J Exp Med 190: 53-64, 1999.
13. Bird JJ, Brown DR, Mullen AC, Moskowitz NH, Mahowald MA, Sider JR, Gajewski TF, Wang C and Reiner SL: Helper T cell differentiation is controlled by the cell cycle. Immunity 9: 229-237, 1998.
14. Deeths MJ, Kedl RM and Mescher MF: $CD8^+$ T cells become non-responsive (anergic) following activation in the presence of costimulation. J Immunol 163: 102-110, 1999.
15. Lalvani AR, Brookes S, Hambleton WJ, Britton AVS, Hill and McMichael AJ: Rapid effector function in $CD8^+$ memory T cells. J Exp Med 186: 859-865, 1997.
16. Perez VL, Parijs LV, Biuckians A, Zheng XX, Strom TB and Abbas AK: Induction of peripheral T cell tolerance *in vivo* requires CTLA-4 engagement. Immunity 6: 411-417, 1997.
17. Sallusto F and Lanzavecchia A: Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. J Med Exp 179: 1109-1118, 1994.
18. Pickl WF, Majdic O, Kohl P, Stockl J, Riedl E, Scheinecker C, Bello-Fernandez C and Knapp W: Molecular and functional characteristics of dendritic cells generated from highly purified $CD14^+$ peripheral blood monocytes. J Immunol 157: 3850-3859, 1996.
19. Fisk B, Hudson JM, Kavanagh J, Wharton JT, Murray JL, Ioannides CG and Kudelka AP: Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res 17: 45-53, 1997.
20. Barbulescu K, Becker C, Schlaak JF, Schmitt E, zum Buschenfelde KM and Neurath MF: Cutting Edge: IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-γ promoter in primary $CD4^+$ T lymphocytes. J Immunol 160: 3642-3647, 1998.
21. Gollob JA, Schnipper CP, Murphy EA, Ritz J and Frank DA: The functional synergy between IL-12 and IL-2 involves p38 mitogen-activated protein kinase and is associated with the augmentation of STAT serine phosphorylation. J Immunol 162: 4472-7781, 1999.
22. Gately MK, Renzetti LM, Magram J, Stern A, Adorini L, Gubler U and Presky DH: The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol 16: 495-521, 1998.
23. Walunas TL, Bakker CY and Bluestone JA: CTLA-4 ligation blocks CD28-dependent T cell activation. J Exp Med 183: 2541-2550, 1996.
24. DeKruyff RH, Gieni RS and Umetsu DT: Antigen-driven but not lipopolysaccharide-driven IL-12 production in macrophages requires triggering of CD40. J Immunol 158: 359-366, 1997.
25. Willerford DM, Chen J, Ferry JA, Davidson L, Ma A and Alt FW: Interleukin-2 receptor α chain regulates the size and content of the peripheral lymphoid compartment. Immunity 3: 521-530, 1996.
26. McDyer JF, Goletz TJ, Thomas E, June CH and Seder RA: CD40 ligand/CD40 stimulation regulates the production of IFN-γ from human peripheral blood monuclear cells in an IL-12- and/or CD28-dependent manner. J Immunol 160: 1701-1707, 1998.
27. Yang Y and Wilson JM: CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. Science 273: 1862-1864, 1996.
28. Walker W, Aste-Amezaga M, Kastelein RA, Trinchieri G and Hunter CA: IL-18 and CD28 use distinct molecular mechanisms to enhance NK cell production of IL-12-induced IFN-γ. J Immunol 162: 5894-5901, 1999.
29. Krummel MF and Allison JP: CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting cells. J Exp Med 183: 2533-2540, 1996.
30. Hemmer B, Stefanova I, Vergelli M, Germain R and Martin R: Relationships among TCR ligand potency, thresholds for effector function elicitation, and the quality of early signaling events in human T cells. J Immunol 160: 5807-5814, 1998.
31. Szabo SJ, Dighe AS, Gubler U and Murphy KM: Regulation of the interleukin (IL)-12Rα2 subunit expression in developing T helper 1 (Th1) and Th2 cells. J Exp Med 185: 815-824, 1997.
32. Perez-Diez A, Butterfield LH, Li L, Chakraborty NG, Economou JS and Mukherji B: Generation of $CD8^+$ and $CD4^+$ T-cell response to dendritic cells genetically engineered to express the MART-1/Melan-A gene. Cancer Res 58: 5305-5309, 1998.
33. Oehen S and Brduscha-Riem K: Differentiation of naive CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. J Immunol 161: 5338-5346, 1998.
34. Jacob J and Baltimore D: Modelling T-cell memory by genetic marking of memory T cells *in vivo*. Nature 399: 593-597, 1999.
35. Guehler SR, Rosalynde JF, Bluestone JA and Barrett TA: Increased threshold for TCR-mediated signaling controls self reactivity of intraepithelial lymphocytes. J Immunol 160: 5341-5346, 1998.
36. Germain RN and Stefanova I: The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. Annu Rev Immunol 17: 467-522, 1999.

Peptide Priming of Cytolytic Activity to HER-2 Epitope 369–377 in Healthy Individuals[1]

Brett W. Anderson, George E. Peoples,[2] James L. Murray, Michael A. Gillogly, David M. Gershenson, and Constantin G. Ioannides[3]

Departments of Gynecologic Oncology [B. W. A., D. M. G., C. G. I.], Surgery [G. E. P.], Bioimmunotherapy, [J. L. M., M. E. G.], and Immunology [C. G. I.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

The presence of tumor-reactive CTLs in tumor infiltrates and in the peripheral blood of cancer patients demonstrates an immune response against tumors that apparently cannot control disease spread. This raises concerns as to whether amplification of this response may be useful during disease progression. Induction of tumor-reactive CTLs in healthy donors at risk, as well as in patients free of disease, may be therapeutically important, based on the hypothesis that CTLs that recognize tumors early may be more effective in containing their progression than CTLs that expand only when the disease progresses. To address the feasibility of priming cytolytic activity in healthy donors, we used the HER-2 peptide E75 (369–377) as an immunogen and autologous peripheral blood mononuclear cell-derived dendritic cells as antigen-presenting cells. We found that of 10 healthy donors tested, two responded at priming with E75 presented on autologous dendritic cells by induction of E75-specific CTL activity. Three other responders were identified after two additional restimulations. Of these five responders, three recognized E75 presented on the ovarian tumor line SKOV3.A2, as demonstrated by cold-target inhibition experiments. Induction of cytolytic activity at priming was enhanced in responders by tumor necrosis factor-α and interleukin 12 but not in the nonresponders. αB7.1 monoclonal antibody added at priming enhanced induction of lytic activity in only one of the four nonresponding donors tested, suggesting that in the majority of donors, E75-precursor CTLs were not tolerized. Because of the possibility that disease may develop in nonresponders, strategies to improve the immunogenicity of tumor antigens for healthy donors may be required for development of cancer vaccines.

INTRODUCTION

Identification of human epithelial tumor Ags,[4] such as the ones expressed on ovarian and breast cancers, allows antitumor vaccination strategies to be developed. Among the most interesting are those that focus on *HER-2* because this proto-oncogene is overexpressed in 20–40% of patients with highly aggressive breast, ovarian, pancreas, colon, and prostate cancers and with consequent poor prognosis. Two clinical trials have targeted *HER-2* (1, 2) using peptides and various adjuvants. The immunogen of choice in these trials was of the HER-2 peptide E75 (369–377, KIFGSLAFL), which maps an epitope frequently recognized by CTLs from tumor-infiltrating/associated lymphocytes of breast and ovarian cancer patients (3, 4).

Although peptide immunization is an appealing approach to tumor immunotherapy because it removes concerns of toxicity and safety while focusing the effectors, the methodology for vaccination and immunological evaluation it is not yet defined (5). Important questions need to be addressed before this approach can be developed to its therapeutic potential. The first question is whether CTLs generated by primary *in vitro* and *in vivo* immunizations will lyse targets endogenously expressing the Ag. It has been shown with model Ag that the majority of peptide-induced CTLs at priming recognized the peptide used as immunogen, but only a small fraction recognized the endogenously presented Ag (6). In some instances, CTLs recognizing endogenously presented Ag could be induced only with a variant peptide (7). Although peptide-specific $CD8^+$ cells may not always be expected to directly lyse tumors *in vitro* and *in vivo*, such cells can recognize peptides derived from extracellular degradation of Ag from dying tumor cells and tumor debris. This may lead to secretion of various cytokine patterns in the tumor environment and conditioning of APCs, resulting in an indirect but significant impact on antitumor responses.

The second concern is how frequently tumor peptide vaccinations induce Ag-specific CTLs in the human population. This concern is attributable to the reported low precursor frequency of tumor-reactive CTLs in healthy individuals, however, this concept has been recently challenged (8, 9). There is also concern over the weak ability of tumor Ag to induce massive Ag-specific CTL expansions, as reported with viral Ags (10).

Received 4/17/00; revised 6/30/00; accepted 8/24/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported in part by Research Grant DAMD17-97-1-7098. Peptide synthesis was supported in part by Core Grant CA 16672.
[2] Present address: Department of Surgery, Uniformed Armed Services, University of the Health Sciences, Bethesda, MD 20814.
[3] To whom requests for reprints should be addressed, at The University of Texas M. D. Anderson Cancer Center, Department of Gynecologic Oncology, 1515 Holcombe Boulevard, Box 67, Houston, TX 77030. Phone: (713) 792-2849; Fax: (713) 792-7586.

[4] The abbreviations used are: Ag, antigen; DC, dendritic cell; *HER-2, HER-2/neu* proto-oncogene; HS, human serum; NP, no peptide; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL, interleukin; mAb, monoclonal antibody; TNF, tumor necrosis factor; LU, lytic unit(s); rVV, recombinant vaccinia vector.

The third concern is whether self-reactive (tumor-reactive) CTLs in healthy donors are silenced by active tolerance or anergy, and stimulation with Ag in peptide form cannot reactivate memory effectors because of B7-CTLA4-mediated peripheral tolerance. Recent studies have shown that induction of melanoma tumor Ag and tumor-reactive CTLs in healthy donors is much less effective than in cancer patients (10). However, induction of tumor-reactive CTLs in healthy donors (as well as in breast and ovarian cancer patients in long-term remission and without evidence of disease) is important based on the hypothesis that CTLs that recognize tumors early may be more effective in containing their progression than CTLs that expand only when the tumor Ag is overexpressed. (11).

Although a number of studies focused on improving the immunogenicity of tumor peptides in selected responding patients and donors using DCs as APCs and inflammatory cytokines, there is little information on the frequency of induction of these responses in unselected healthy donors. However, this question is important because cancer vaccines are expected to be given to distinct individuals, of which some may be at risk to develop disease, whereas others may be free of disease and otherwise considered healthy individuals. Thus, the frequency of CTL responses to a tumor Ag in the population becomes an important issue. We rationalized that if the frequency of responses to E75 priming is similar to or lower than the frequency of responses to MART-1 (10), initial screening of a large panel of healthy donors may identify at least one responder. Cells of this responder can be then used as positive controls to address the questions of costimulation and of cytokine help in elicitation of cytolytic function in nonresponders.

We developed a model for priming T cells of PBMCs from healthy donors with the HER-2 peptide E75. We used as APCs autologous DCs, always freshly generated in the presence of GM-CSF + IL-4 from the same PBMC sample. To determine the role of costimulation in this system, αB7.1 antibodies were added at priming. To establish whether IL-12 and TNF-α are essential for CTL priming, stimulations were performed in the presence or absence of these cytokines. We found that 2 of 10 healthy donors responded by inducing E75-specific cytolysis at peptide priming and 5 of 10 at restimulation. Although IL-12 and TFN-α potentiated CTL induction in the responsive donors, they did not help induce CTLs in nonresponders, suggesting that additional factors to the nature of APCs and inflammatory cytokine conditioning regulate the induction of CTLs specific for *HER-2* by synthetic peptides.

MATERIALS AND METHODS

Cells, Antibodies and Cytokines. HLA-A2$^+$ PBMCs were obtained from healthy volunteers from the Blood Bank of M. D. Anderson Cancer Center. The HLA phenotypes of the donors used in this study were: donor 1 (A2, B7, 44); donor 2 (A2, 33, B40, 44); donor 3 (A2, 33, B41, 81); donor 4 (A1, 2, B27, 44); donor 5 (A1, 2 B44, 57, Cw5, 6); and donor 6 (A2, 31, B35, 44, Cw4, w5). For the other four donors only, the HLA-A2 expression was determined. T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (3, 4). mAb to CD3, CD4, CD8 (Ortho), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and B7.2 (CD80 and CD86, Calbiochem), intercellular adhesion molecule-1 (ICAM-1 CD54; Calbiochem), CD40L (Ancell, Bayport, MN), HLA-A2 (clone BB7.2; American Type Culture Collection), and MHC-II (L243; Dako Corp., Carpinteria, CA) were used as unconjugated, FITC, or phycoerythrin conjugated. The following cytokines were used: GM-CSF (Immunex Corp., Washington, DC; specific activity, 1.25×10^7 colony-forming units/250 mg); TNF-*a* (Cetus Corp., Emeryville, CA; specific activity, 2.25×10^7 units/mg); IL-4 (Biosource International; specific activity, 2×10^6 units/mg); and IL-2 (Cetus Corp.; specific activity, 18×10^6 IU/mg). IL-12 at 5×10^6 units/mg was a kind gift from Dr. Stanley Wolf (Department of Immunology, Genetics Institute, Cambridge, MA).

Synthetic Peptides. The HER-2 peptides used were: E75 (369–377) and the unnatural modified Muc-1 peptides D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The corresponding natural peptides do not bind HLA-A2. Introduction of an HLA-A2 anchor and sequence modification in Muc1 in residues contacting TCR lead to an unnatural sequence (12). All peptides were prepared by the Synthetic Antigen Laboratory of M. D. Anderson Cancer Center and purified by high-performance liquid chromatography. Peptides were 95–97% pure by amino acid analysis. Peptides were dissolved in PBS and stored frozen at −20°C in aliquots of 2 mg/ml Polyglycol bead-containing E75 were a kind gift of Dr. Kenneth Grabstein (Corixa Corp., Seattle, WA).

Immunofluorescence. Antigen expression by DCs and T cells was determined by fluorescence-activated cell sorter using a flow cytometer (EPICS Profile Analyzer; Coulter Co., Hialeah, FL). DCs were defined by the presence of CD13 and absence of CD14 marker after culture in GM-CSF and IL-4. For phenotype analysis, DCs were incubated with phycoerythrin-conjugated anti-CD13 mAb and FITC-conjugated mAb specific for a surface Ag.

Generation of PBMC-derived DCs. CD13$^+$ DCs were generated from freshly isolated PBMCs by following the established CD14 methods (13, 14). Complete RPMI 1640 (containing 10% FCS) supplemented with 1000 units/ml GM-CSF, and 500 units/ml IL-4 were added to each well containing plastic-adherent cells, changed every 48 h, and maintained for 7 days. In separate studies, performed in parallel, we attempted to grow DCs in medium containing either HS or in AIM-V medium. The growth and expression of surface markers of DC cultured in complete RPMI 1640 was significantly better than of DCs cultured in other conditions; thus, in this study only DCs cultured in complete RPMI 1640 were used. CD8$^+$ cells were isolated by removing first the CD4$^+$ and then the CD16$^+$ and CD56$^+$ cells from the nonadherent population using Dynabeads (Dynal, Oslo, Norway). After depletion, the resulting cells were 97% CD8$^+$, as determined by flow cytometry.

T-Cell Stimulation by Peptide-pulsed DCs. DCs were washed three times in serum-free medium, plated at 1.2×10^5 cell/well in 24-well culture plates, and pulsed with peptides at 25–50 μg/ml in serum-free medium for 4 h before the addition of responders. TNF-α (50 units/ml) was added to DCs for the last hour to stimulate Ag uptake and presentation (13). Autologous PBMCs or isolated CD8$^+$ cells in RPMI 1640 containing 10% HS were added to DCs at 1.5×10^6/ml, followed 60 min later by IL-12 at 3 IU/ml, IL-2 was added 16 h later to each well at 60 IU/ml and every 48 h thereafter. For inhibition studies,

Table 1 Summary of E75-specific cytolytic responses in healthy donors

Donor no.	E75-specific response at primary	Enhancement by IL-12/ TNF-α	E75-specific CTL at restimulation[a]	Specific tumor lysis	No. of independent priming experiments
1	+	+	+ (1)[b]	+	6
2	−	−	− (2)	ND	3
3	−	−	+[c] (3)	+	6
4	−	−	− (3)	ND	4
5	+	+	+ (1)	+	4
6	−	−	+[c] (3)	−	5
7	±[d]	±	+ (1)	−	4
8	−	−	− (2)	ND	4
9	−[e]	−	− (2)	ND	3
10	−	−	− (1)	ND	1
Total	2/10	2/10	5/10	3/5	40

[a] +, specific lysis of E75-pulsed T2 was ≥20% at an E:T ratio of 20:1; −, specific lysis of E75-pulsed T2 was not significantly different than lysis of control T2 cells pulsed with no peptide. ND, not done.

[b] Numbers in parentheses, number of restimulations.

[c] E75-specific CTL activity was detected after four stimulations.

[d] E75-specific CTL activity was ≤10% at an E:T of 10:1.

[e] E75-specific activity, observed occasionally, could not be enhanced by peptide plus cytokine stimulation but was enhanced by αB7.1.

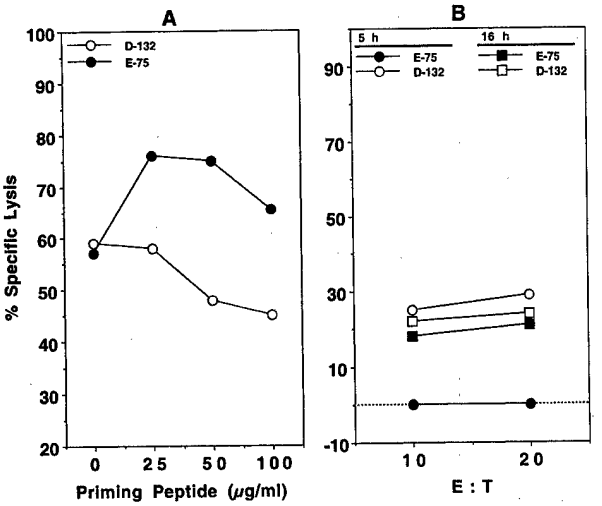


Fig. 1 *A*, induction of E75-specific cytolytic activity at priming with various concentrations of E75. Plastic nonadherent PBMCs from donor 1 were stimulated with the indicated concentrations of E75. Seven days later, cells were removed from cultures and tested for CTL activity against T2 pulsed with 25 μg/ml E75 or D132. The experiment was performed in triplicate at a 20:1 ratio from all stimulation groups. Differences between T2-E75 (●) recognition and T2-D132 recognition (○) are significant ($P < 0.05$) in all DC-E75 stimulation groups. *B*, priming of PBMCs from donor 9 (nonresponder) with E75 in polyglycol beads does not induce E75-specific CTL activity. Experimental conditions were as described for donor 1.

mAbs specific for B7.1, B7.2, and HLA-A2 were added to DCs 1 h before responders in amounts reported to be inhibitory by the manufacturers.

CTL and Cytokine Assays. Recognition of peptides used as immunogens by CTLs was performed as described (3, 15). Equal numbers of viable effectors from each well were used in all assays. To minimize cross-reactive recognition of human peptides, all stimulations were preformed in medium containing HS, whereas CTL assays were performed using medium containing FCS (15). This minimized background cytolysis attributable to activation of other autoreactive cells to Ags present in HS. Specific LU were determined as described (10, 16) and are expressed as LU $30/10^7$ cells.

RESULTS

Priming of Healthy Donors PBMCs with E75-pulsed Autologous CD14-derived DCs. To address the question of whether priming with E75 presented on DCs induce E75-specific CTL activity, plastic nonadherent PBMCs from healthy donors were stimulated with autologous DCs generated by culture in GM-CSF + IL-4. DCs were pulsed with E75 at 25–50 μg/ml. Seven days later, cytolytic activity was determined against E75-pulsed T2 using as control T2 cells that were not pulsed with peptide. Cumulative results are presented in Table 1. The results show that of 10 healthy donors tested, only 2 showed stable and consistent recognition of E75 after priming with DC-E75. These cumulative results show that E75 can induce specific cytolytic activity at priming only in a small fraction of healthy donors (2 of 10; 20%). These results were confirmed because each CTL induction experiment was repeated at least three times at different time points and always with freshly isolated PBMCs (except donor 10). In 2 donors (donors 2 and 9), specific CTL activity was occasionally detected in one of three independent experiments, but this activity was unstable and could not be further expanded. Induction of this activity in donor 9 required αB7.1 at priming (shown in Fig. 5). In contrast, in the two responding donors, specific CTL activity was detected at priming in 4 of 6 (donor 1) and 3 of 4 (donor 5) independently performed induction experiments over periods of 1 year and 6 months, respectively.

To address the question of the optimal Ag concentration for priming, autologous DCs were pulsed with 0, 25, 50, and 100 μg/ml of E75 prior to the addition of responders. The resulting cultures were supplemented with TNF-α and IL-12 at priming and IL-2 every 48 h for the following 7 days. A representative experiment of the specificity of recognition of CTLs from donor 1 primed by DC-E75 is shown in Fig. 1. The results show that the specificity of recognition of CTLs induced by E75 at three concentrations of peptide (25, 50, and 100 μg/ml) was similar, with 50 μg/ml E75 inducing the highest differences in lytic activity between targets pulsed with E75 or targets pulsed with no peptide. CTLs could not be induced even by high concentrations of E75 in any of the nonresponders. To address whether E75-stimulatory potency was increased by delivery of Ag encapsulated in polyglycol beads, PBMCs from two nonresponders (numbers 2 and 9) were stimulated with autologous DCs pulsed with polyglycol beads in numbers to generate equivalent E75 concentrations with peptides. The results are shown for donor 9 (Fig. 1*B*). Stimulation with encapsulated-E75 failed to induce specific CTL activity at priming, as concluded from 5-h CTL assays. However, in longer CTL assays (16 h), lysis of T2-E75 pulsed targets increased significantly compared with

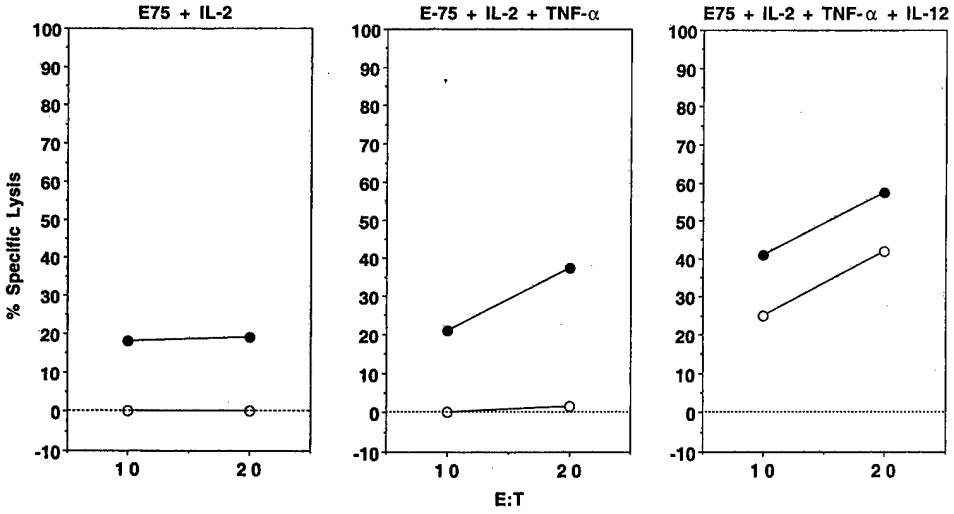


Fig. 2 Induction of E75-specific cytolytic activity at priming in donor 5. Plastic nonadherent PBMCs were stimulated with 50 μg/ml E75 pulsed on autologous DCs. TNF-α (50 IU/ml), IL-12 (3 IU/ml), and IL-2 (60 IU/ml) were added to these cultures. Differences in lysis of T2 cells pulsed with E75 (●) or not (○) are significant in all groups ($P < 0.02$). All experiments were performed simultaneously in triplicate. Results of one experiment of two performed with similar results are shown.

that of control targets, and the levels of recognition were similar. This suggested that either the numbers of E75-specific CTLs were low, or CTLs in these two donors had weak cytolytic activity. It should be mentioned that in both experiments, IL-2 was added at 48 and 96 h in higher concentrations (150–200 IU/ml) to facilitate T-cell expansion. Although this increased the background lysis, it did not change the patterns of recognition.

Effects of TNF-α and IL-12 in Induction of CTL Activity at Priming. Both TNF-α and IL-12 have been described in different systems to augment cytotoxicity of $CD8^+$ cells (17, 18). To determine whether the E75-induced CTL activity could be enhanced by TNF-α and IL-12, CTL priming experiments were performed in the presence and absence of these cytokines (Fig. 2). The results from donor 5 show that the addition of TNF-α at the time of pulsing with peptide-enhanced, E75-specific activity compared with cultures that received only IL-2. When IL-12 was added, the increase in T2-E75 killing was paralleled by an increase in nonspecific killing. The addition of IL-12 at higher concentrations during priming did not increase the specific but rather the nonspecific CTL activity. This was equally true when isolated $CD8^+$ cells were used as effectors (data not shown). To address whether these cytokines enhanced cytolytic activity at priming in nonresponders, the experiment was repeated with donor 1 (responder) and donor 4 (nonresponder). The results in Fig. 3 show that TNF-α increased specific CTL activity in donor 1 but not in donor 4. Although T2-E75 lysis increased in TNF-α-treated cultures, it was still not significantly different from the control. Similar to donor 5 (in both donors 1 and 4), IL-12 increased both the nonspecific and specific lysis. These results were confirmed with all donors tested (Table 1). IL-12 alone, or together with IL-2 and TNF-α, failed to induce specific CTL activity in nonresponders.

To address whether the increase in cytolytic activity induced by TNF-α and IL-12 at priming was attributable to changes in the levels of $CD8^+$ cells in these cultures, we determined the percentages of $CD8^+$ and $CD4^+$ in E75-primed cultures from donor 5 used in the experiment shown in Fig. 2. The results in Table 2 show only small differences between the

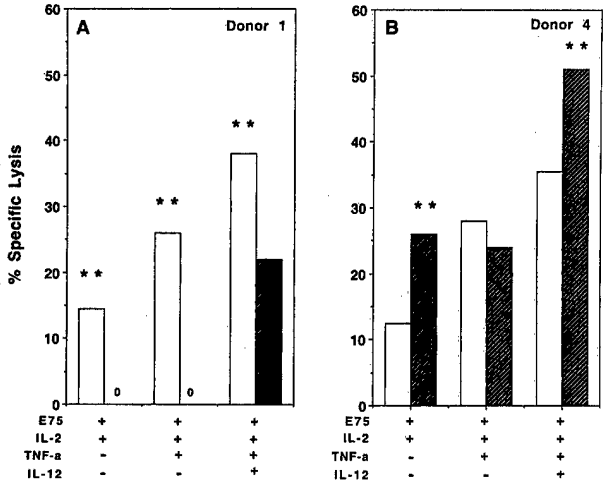


Fig. 3 Addition of IL-12 at priming does not induce specific CTL activity in the nonresponders. Donor 1 (responder; *A*) and donor 4 (nonresponder; *B*) were tested in the same experiment. The E:T ratio was 20:1. For donor 1, this experiment was performed at a different date with a fresh blood sample than the experiment shown in Fig. 1. *A:* **, significant differences in recognition of E75 (□) compared with control NP (■; $P < 0.02$). *B:* **, significantly higher recognition of T2-NP than of T2-E75 ($P < 0.05$).

percentage of $CD8^+$ cells in cultures containing IL-2 only compared with IL-2 + TNF-α or IL-2 + TNF-α + IL-12. A caveat of this analysis is that E75 tetramers are not yet available; thus, we could not determine whether TNF-α and IL-12 increased the numbers of E75-specific cells in these cultures.

Involvement of B7-CD28 Costimulation in E75-specific CTL Priming. To address whether induction of E75-specific cytolytic activity required costimulation, αB7.1 and αB7.2 mAbs were added at priming. The results in Fig. 4 show that αB7.1 significantly inhibited induction of specific CTL activity in donor 5 (by >80%), whereas αB7.2 had a much smaller effect.

Table 2 Expression of CD8 and CD4 on E75-primed cultures

Peptide	IL-2	TNF-α	IL-12	%CD8$^+$	%CD4
−	+	−	−	21.3	59.1
+	+	−	−	18.9	64.9
−	+	+	−	N.D.	N.D.
+	+	+	−	21.4	63.4
−	+	+	+	25.2	55.9
+	+	+	+	25.1	55.0

Plastic-nonadherent PBMCs from donor 5 were stimulated with autologous DCs pulsed with E75, or as control with autologous DCs that were not pulsed with E75. TNF-α and IL-12 were added at priming, whereas IL-2 was added 24 h later. Cells were stained with the corresponding α-CD8 and α-CD4 in antibody 7 days later and examined by fluorescence-activated cell sorter.

B7.2 was expressed at significantly higher levels than B7.1 on DCs. Furthermore, interaction of DCs with T cells and cytokines was paralleled by B7.2 but not B7.1 up-regulation within 20 h (not shown), raising the possibility that αB7.2 mAb was insufficient for blocking. The strong inhibition of E75-specific CTL induction by αB7.1 suggested that the responder CTLs in this donor are more likely naïve T cells.

To determine whether the nonresponders were activated but tolerized T cells, which cannot expand because of B7-CTLA4 interaction, the experiment was repeated with four nonresponders (nos. 3- 6) using the same amounts of αB7.1 as in donor 5. The results are shown in Fig. 5. In donor 9, the addition of αB7.1 at priming led to induction of specific CTL activity (Fig. 5*A*). It is tempting to speculate that in this donor, activated but tolerized E75-specific CTLs were present, and they cannot expand because of the B7-CTLA4 interaction. Additional studies are needed to address this point. In donors 4 and 5, the addition of αB7.1 at priming failed to induce significant specific cytolytic activity. These results were confirmed with donor 3 (not shown). Thus, of five donors tested, αB7.1 inhibited E75-specific CTL priming in one (no. 5), enhanced CTL priming in another one (no. 9), but failed to enhance induction of specific CTL activity in three (nos. 3–5). These experiments were repeated, and the results were confirmed. Thus, the requirements for B7-CD28 costimulation appeared to be dependent on the donor.

Induction of CTL Activity at Restimulation. To address whether E75 restimulation enhanced specific cytolytic activity, E75 primed PBMCs from all donors were restimulated with DC-E75. Of the eight nonresponders at primary stimulation, only three (donor nos. 3, 6, and 7) increased their E75-specific lysis at restimulation. In two of three donors (donors 3 and 6), specific E75 recognition was borderline after restimulation. E75-specific cytolysis was observed at the fourth stimulation with DC-E75 in these two donors (data not shown). In the other five donors that were both primed and restimulated with E75 but failed to show specific CTL activity, additional restimulations were not attempted, because of the low levels of recognition of T2-E75 at restimulation compared with nonspecific lysis. We rationalized that the five nonresponders will require a minimum of four and even more restimulations to possibly elicit E75-specific CTLs. Thus, even if cytolytic activity would be detected after four to five stimulations, this finding would also support the hypothesis of weak E75 immunogenicity in these individuals.

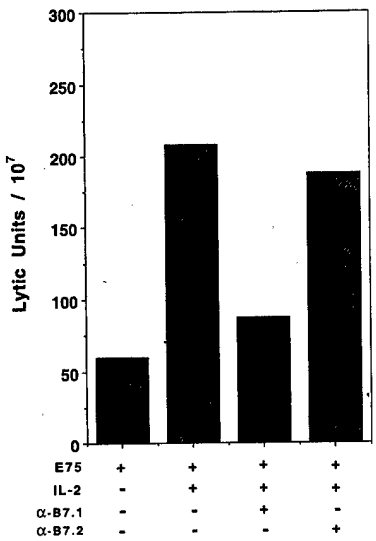


Fig. 4 Priming of E75-specific cytolysis in donor 5 requires B7 costimulation. Plastic nonadherent PBMCs from donor 5 were primed with E75 in the presence or absence of αB7.1 and αB7.2 mAb. Results are shown as specific LU calculated from the percentage of specific lysis against E75 and against the specificity control, the unnatural negative control peptide D132 (10, 12) at two E:T ratios (10:1 and 20:1). D132 was used as control to stabilize HLA-A2 on T2 cells similarly with E75.

Recognition of Tumor Cells by E75-primed CTLs. To address the question of whether E75-primed CTLs from healthy donors recognized endogenously presented epitopes, CTLs generated from donors 1, 3, 5, 6, and 7, which showed peptide-specific lytic activity, were tested for their ability to lyse the tumor line SKOV3.A2 and its A2$^-$ counterpart, SKOV3. Except for HLA-A2, all other histocompatibility Ags on SKOV3 and SKOV3.A2 are identical. To verify that the responses are E75 specific, cold-target inhibition experiments using unlabeled T2-E75 as specific target and T2-NP as negative control were performed in parallel. T2 express only HLA-A2 and low levels of HLA-B5. The results are summarized in Table 1. Peptide-specific CTLs from donors 6 and 7 did not show specific recognition of SKOV3.A2 tumor. However, E75-specific CTLs from donors 1, 3, and 5 recognized endogenous E75. These donors do not express HLA-B5, suggesting that E75 was presented by HLA-A2. It should be mentioned that E75-specific CTLs were induced in donors 1 and 5 at priming with DC-E75, whereas in donor 3, expression of this cytolytic activity required four stimulations with DC-E75. These results indicated that of 10 healthy donors tested, only three (33%) responded by inducing CTLs that specifically recognized tumor cells. This percentage was higher in the group of responders with peptide-specific CTLs (three of five; 60%). The results with donor 5 are shown in Fig. 6, *A* and *B*. Both E75-primed cultures from donor 5 lysed SKOV3.A2 better than SKOV3, suggesting that they recognize an epitope associated with HLA-A2. To address whether these cultures recognized an endogenous presented epitope similar to E75, we performed cold-target inhibition experiments. The results in Fig. 6*C* show that T2-E75 significantly inhibited by >50% recognition of SKOV3.A2 by CTLs from donor 5 com-

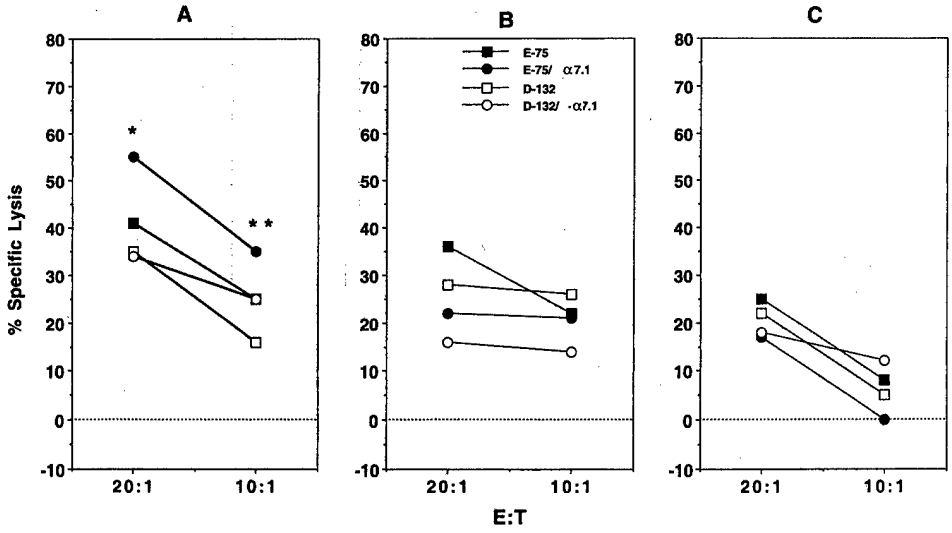


Fig. 5 Effects of B7.1 blocking on priming of E75-peptide-specific cytolytic activity in distinct healthy donors. Induction of E75-specific cytolysis by blocking of B7.1 in donor 9 (*A*) but not in donors 3 and 4 (*B* and *C*). αB7.1 was added to DCs 30 min before the addition of responders. Cultures were primed with E75 in the presence (●) or absence (□, ■) of αB7.1 in the same experiment. Cytolytic activity was determined in the same experiment in triplicate against targets pulsed with E75 (■, ●) or the control unnatural peptide D132 (□, ○). D132 was used to stabilize HLA-A2 at comparable levels with E75, thus minimizing the natural killer-derived background lysis. * and **, $P < 0.01$ and <0.05 respectively, compared with the lysis of T2-D132.

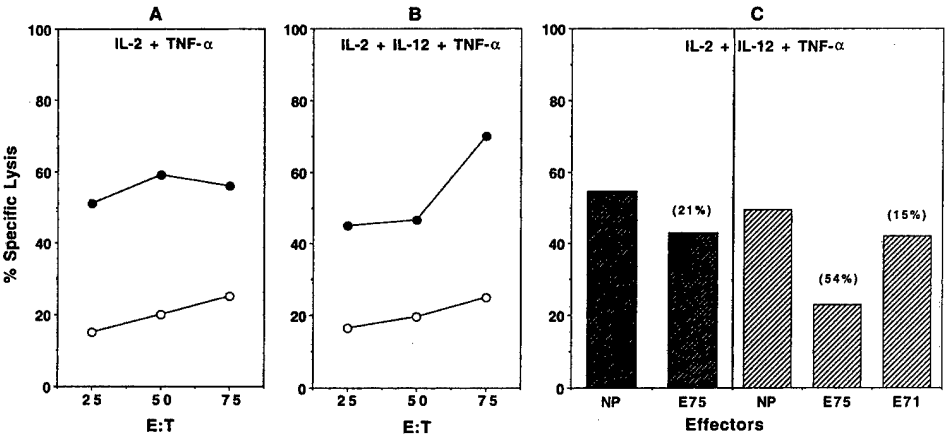


Fig. 6 *A* and *B*, E75-primed PBMCs from donor 5 recognize SKOV3.A2 (●) better than SKOV3 (○) cells, suggesting that they recognize an HLA-A2-associated Ag. Differences between the levels of lysis of two targets are significant ($P < 0.05$). *C*, cold-targeted inhibition of SKOV3.A2 lysis by E75 + IL-2 + TNF-α-induced cells by T2-E75, but not by negative control T2-NP, or the negative peptide control T2-E71 demonstrate E75-specific recognition for donor 5 (■) and donor 1 (□). Differences in lysis of SKOV3.A2 cells incubated with T2-NP (percentage of specific lysis: 56, 59, and 60%) and T2-E75 (percentage of specific lysis: 39, 42, and 44%) were significant ($P < 0.05$).

pared with control T2-NP, which expressed HLA-A2. This inhibition was peptide specific because it was not observed with the control peptide E71 pulsed on T2, suggesting that some E75-primed CTLs recognized an endogenously presented epitope, but these cells are not the majority in the effector population. Similar results were obtained with E75-primed cells from donors 1 (Fig. 6*C*) and 3 (not shown). However, the levels of cold-target inhibition were lower and ranged between 20 and 25% in two separate experiments. This suggested that only a subpopulation of E75-induced CTLs recognize endogenous generated epitopes. Thus, successful induction of E75-specific CTL activity at priming with E75 using DCs as APCs and inflammatory cytokine support appears to be dependent of additional factors other than the nature of APCs and B7 costimulation.

DISCUSSION

In this study, we investigated the ability of the HER-2 peptide E75 to prime E75-specific cytolytic activity in healthy donors when presented on autologous DCs. We found that only

2 of the 10 HLA-A2$^+$ healthy donors tested responded by induction of E75-specific cytolytic activity at priming. This was confirmed in replicated experiments performed over time, and the use of various cytokine combinations IL-2+IL-12, IL-2 + TNF-α + IL-12, or preculture in IL-2, preculture in IL-2 + RANTES.[5] These results indicated that E75-specific or cross-reactive T cells endowed with cytolytic activity can be elicited at priming in only a fraction of healthy donors (20%) but induced in an additional 30%. Of interest, E75-primed CTLs from these two donors recognized E75 presented on tumor cells, because their activity was inhibited in cold-target inhibition assays.

Two cytokines, TNF-α and IL-12, were used to potentiate E75-specific CTL induction. TNF-α has been described to increase Ag uptake and presentation by DCs (13) and to potentiate CTL generation in animal models (17). IL-12 has also been described to potentiate CTL induction and cytolytic activity (18, 19). TNF-α and IL-12 increased the levels of cytolytic activity in responders but had no effect in nonresponders. This suggests that these cytokines are not essential for priming of E75-specific CTL activity. Of interest, in the responders, induction of E75-specific cytolytic activity was inhibited by αB7.1, suggesting a requirement for costimulation in the induction of cytolytic activity as described (20) for other Ag raising the possibility of DC-E75 primed naïve T cells.

Induction of E75-specific cytolytic activity at priming with peptide observed with two healthy donors is of interest in evaluating the potential of this epitope for tumor-specific CTL induction and cancer vaccine development. In human tumor systems (most instances), priming with peptide required several repeated stimulations of healthy donor PBMCs before specific cytolysis was detected. CTLs specific for tyrosinase 369–377 peptide were detected in four of five healthy donors after three restimulations with peptide (21). Peptide-specific CTLs were induced in healthy donors using DCs and peptides from gp100, tyrosinase, and MART-1/MelanA. Detection of CTL activity required three to four cycles of stimulation (22). Similarly, presentation of MART-1 by DCs transduced with an adenoviral vector construct carrying the *MART-1* gene required three stimulations for induction of specific cytolysis (23), although in some donors, specific cytolytic effectors were detectable 7 days after priming (24). In contrast, in another study, MART-1 (27–35)-specific cytolytic function could be induced in a nonresponder only, using APCs infected with rVVs expressing rVV-B71/2 + peptide, or rVV-B7.1 + MART-1 and restimulated with peptide, but not by peptide stimulation only (25, 26). Thus, the potency of E75 to induce cytolytic function in healthy donors appears similar to that of the MART-1 peptide 27–35.

Few studies have investigated the frequency of responses to tumor Ags in healthy donors at priming and restimulation or the consistency of these responses for an individual. This aspect is important because of its implications for protective vaccination in healthy donors or ovarian, breast, and prostate cancer patients without evidence of disease. In one extensive study, Marincola *et al.* (10) found that after several stimulations with MART-1 (27–35), five of nine healthy donors (56%) responded by induction of specific cytolytic effectors. Only one to two donors showed weak activation of cytolysis at priming. In an independent study, 4 of 16 healthy donors (25%) responded to MART-1 (27–35) after two stimulations (27). Similarly, anti-p53 (264–272) cytolytic effectors were generated from 2 of 5 healthy donors (40%) after several restimulations with peptide-pulsed DCs (28), whereas anti-gp100 CTLs were elicited in 1 of 10 healthy donors at priming (29).

In contrast with melanoma Ag, the immunogenicity of which has been repeatedly investigated in extensive studies, the experience with E75 is limited. Similar to the MART-1 peptide, E75 activated rapid cytokine secretion from cultured ovarian tumor-infiltrating lymphocytes or CTL lines (30, 31) and activated cytolysis in tumor-associated lymphocytes (32). Freshly isolated PBMCs from cancer patients that were not vaccinated with peptide rapidly responded to E75 and to another HER-2-epitope, GP2 (33), in a similar fashion as melanoma patients to MART-1, by induction of specific cytolysis and tumor recognition (34). The ease by which E75- and GP2-specific cytolytic activities were induced in patients suggested that E75 and GP2 reactivated effector/memory CTLs rather than primed naïve cells (34). Our results showing 2 of 10 responders at priming (20%) and 5 of 10 responders at restimulation (50%) indicate that E75 is similar to MART-1 (27–35), tyrosinase (369–377), and p53 (264–272) in its ability to activate cytolysis in randomly selected healthy donors.

It is possible that E75 cannot elicit a complete response in all donors during PBMC priming. Our recent studies on cytokine responses by E75-primed PBMCs in healthy donors show that E75 rapidly activated specific IFN-γ release in five of six healthy donors, an effect that was enhanced by IL-12 (36). Although the same donors were used in these studies and IL-12 was used in parallel experiments, we could not observe a similar effect with respect to induction of cytolysis. Thus, a complete response (cytokines and cytolysis) was observed only in two donors of the six tested. This suggests that E75 may act as a partial agonist. In support of this possibility, Zaks and Rosenberg (1) reported recently that E75 vaccination in incomplete freund adjuvant of four cancer patients led to a peptide-specific response at restimulation in all patients (cytolysis and IFN-γ). T cells from two of three E75-vaccinated patients recognized, occasionally, tumor cells by specific IFN-γ secretion but failed to show specific tumor lysis (1). Similarly, tyrosinase 369–377-specific CTLs from two of four responders failed to recognize tyrosinase-expressing tumors (21). Preliminary results from a vaccine trial in breast and ovarian cancer patients indicate that PBMCs from only two of six E75-vaccinated patients (33%) stimulated *in vitro* elicited specific CTL activity against peptide and specific tumor recognition, although all responded to E75 by specific IFN-γ induction (2).

The similar response rates for induction of cytolysis in healthy donors to *in vitro* tumor peptide vaccination raise a number of questions about the application of this approach:

(*a*) Why, regardless of the Ag used, only 10–20% of healthy donors respond at priming, and only 40–50% at restimulation with peptide? One possibility is that healthy donors have different precursor frequencies for the CTL epitopes, and repeated peptide stimulation (three to four times) is not sufficient to expand the

[5] T. V. Lee and C. G. Ioannides, preliminary data.

effector population to sufficiently high numbers to detect CTL activity. Thus, one approach is to continue repeated vaccinations and deliver exogenous help by helper peptides and cytokines until such CTL responses are elicited (9, 35).

(*b*) If the 10–20% of donors that respond to peptide priming have higher pCTL frequencies for E75/MART-1 than nonresponders, then what is the reason for this increased frequency? It is tempting to speculate that local inflammatory conditions and cross-reactive priming may activate CTL precursors, such as in donor 9, and these precursors become tolerized.

(*c*) If pCTL frequency is similarly low in all healthy donors, then why do some respond better than others? One possibility is that discrete changes in HLA-A2 attributable to HLA-A2 polymorphism may lead to a more immunogenic E75 in some donors. In support of this possibility, Maurer *et al.* (37) demonstrated that mutated HLA-A2 in position 97 can segregate MART-1 (27–35)-induced cytolysis from cytokine production. Furthermore, the pool of E75 precursors may expand or contract over time because of different environmental factors (38, 39). This may be supported by the fact that the responders showed E75-specific CTL activity frequently, whereas some of the nonresponders showed activity only occasionally in the majority of independently performed experiments. Additional studies using carboxyfluorescein acetate to determine cell division, E75-tetramers to determine the frequency of E75-specific CTLs, and intracellular IFN-γ staining are required to distinguish among these possibilities.

Increased HLA-A2 binding affinity by COOH-terminal modification was able to enhance tumor Ag immunogenicity (in some instances), as shown in our previous studies (15) and by other investigators using the melanoma Ag gp100 (40, 41). However, increased HLA-A2 binding affinity does not always predict a higher T-cell receptor signaling or a complete T-cell activation (reviewed in Ref. 42). In some of the reported cases, CTLs induced by higher HLA-A2 affinity binding variant showed low affinity for the tumor cells (15), and more recently (40, 41), some reports have suggested that they preferentially targeted tumors expressing high numbers of the epitope. Thus, novel immunogens need to be designed with emphasis on modifications in the Ag that would induce high rates of proliferation and select responders of high cytolytic activity, *i.e.*, high catalytic activity as demonstrated by enzymes. Because restimulations may induce apoptosis, it remains to be seen whether tumor-specific CTL expansion would require several agonists, each being capable of activating one effector function at a time. At the present, the results of this study demonstrated that cytolytic effectors to an epitope on HER-2, which is overexpressed on the majority of epithelial tumors, can be elicited in a fraction of healthy individuals at priming, and in nonresponders, the precursors were not tolerized. Because these studies were performed with 10 donors and substantiated in multiple replicate induction experiments using the same stimulation system, this suggests that this stimulation system may be identifying individuals that will respond to vaccine with a tumor Ag. This may have implications for preventative vaccination in high-risk individuals.

ACKNOWLEDGMENTS

We thank the Blood Bank personnel for donating blood repeatedly for this project.

REFERENCES

1. Zaks, T. Z., Rosenberg, S. A. Immunization with a peptide epitope (p369–377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu$^+$ tumors. Cancer Res., *58:* 4902–4908, 1998.

2. Anderson, B., Lu, J. J., Ibrahim, N., Hortobagyi, G., Brewer, H., Przepiorka, D., Ioannides, C., Grabstein, K., Cheever, M., and Murray, J. Immunomodulating effects of a HER2/neu derived HLA-A2 specific peptide E75 combined with GM-CSF in patients with metastatic breast and ovarian cancer. Proc. Am. Soc. Clin. Oncol., *18:* A1691, 1999.

3. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of *HER-2/neu* protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J. Exp. Med., *181:* 2109–2117, 1995.

4. Hudson, J. M., Castilleja, A., Murray, J. L., Honda, T., Kudelka, A., Singletary, E., Wharton, J. T., and Ioannides, C. G. Growth and antigen recognition of tumor-infiltrating lymphocytes from human breast cancer. J. Interferon Cytokine Res., *18:* 529–536, 1998.

5. Dudley, M. E., Nishimura, M., I., Holt, A. K. C., and Rosenberg, S. A. Antitumor immunization with a minimal peptide epitope (G9–209-2M) leads to a functionally heterogeneous CTL response. J. Immunother., *22:* 288–298, 1999.

6. Carbone, E. R., Moore, M. W., Sheil, J. M., and Bevan, M. J. Induction of cytotoxic T lymphocytes by primary *in vivo* stimulation with peptides. J. Exp. Med., *167:* 1767–1779, 1988.

7. Kelly, J. M., Sterry, S. J., Cose, S., Turner, S. J., Fecondo, J., Rodda, S., Fink, P. J., and Carbone, F. R. Identification of conserved T cell receptor CDR3 residues contacting known exposed peptide side chains from a major histocompatibility complex class I-bound determinant. Eur. J. Immunol., *23:* 3318–3326, 1993.

8. Pittet, M., Valmori, D., Dunbar, P. R., Speiser, D. E., Lienard, D., Lejeune, F., Fleischhauer, K., Cerundolo, V., Cerottini, J-C., and Romero, P. High frequencies of naïve melan-A/MART-1-specific CD8$^+$ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2-individuals. J. Exp. Med., *190:* 705–715, 1999.

9. Anichini, A., Molla, A., Mortarini, R., Tragni, G., Bersani, I., DiNicola, M., Gianni, A. M., Pilotti, S., Dunbar, R., Cerundolo, V., and Parmiani, G. An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. J. Exp. Med., *190:* 651–667, 1999.

10. Marincola, F. M., Rivoltini, L., Salgaller, M. L., Player, M., and Rosenburg, S. A. Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence of *in vivo* priming by tumor cells. J. Immunother., *19:* 266–277, 1996.

11. Alexander-Miller, M. A., Leggatt, G. R., and Berzofsky, J. A. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc. Natl. Acad. Sci., *93:* 4102–4107, 1996.

12. Ioannides, C. G., Fisk, B., Jerome, K. R., Chesak, B., Irimura, T., Wharton, J. T., and Finn, O. J. Ovarian tumors reacting cytotoxic T lymphocytes can recognize peptide determinants on polymorphic epithelial mucins. *In:* F. Sharp, P. Mason, J. Berek, and A. Blackett (eds.), Ovarian Cancer, pp. 317–330. London: Chapman and Hall, 1994.

13. Sallusto, F., and Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor. J. Med. Exp., *179:* 1109–1118, 1994.

14. Pickl, W. F., Majdic, O., Kohl, P., Stockl, J., Riedl, E., Scheinecker, C., Bello-Fernandez, C., and Knapp, W. Molecular and functional characteristics of dendritic cells generated from highly purified CD14$^+$ peripheral blood monocytes. J. Immunol., *157:* 3850–3859, 1996.

15. Fisk, B., Chessak, B., Pollack, M., Wharton, J. T., and Ioannides, C. G. Oligopeptide induction of a cytotoxic T lymphocyte response to *HER-2/neu* proto-oncogene *in vitro*. Cell. Immunol., *157:* 412–427, 1994.

16. Ioannides, C. G., Platsoucas, C. D., Rashed, S., Wharton, J. T., Edwards, C. L., and Freedman, R. S. Tumor cytolysis by lymphocytes infiltrating ovarian malignant ascites. Cancer Res., *51:* 4257–4265, 1991.

17. Gorelik, L., Bar-Dagan, Y., and Mokyr, M. B. Insight into the mechanism(s) through which TNF promotes the generation of T cell-mediated antitumor cytotoxicity by tumor bearer splenic cells. J. Immunol., *156:* 4298–4308, 1996.

18. Mehrotra, P. T., Wu, D., Crim, J. A., Mostowski, H. S., and Siegel, J. P. Effects of IL-12 on the generation of cytotoxic activity in human $CD8^+$ T lymphocytes. J. Immunol., *151:* 2444–2452, 1993.

19. Bhardwaj, N., Seder, R. A., Reddy, A., and Feldman, M. V. IL-12 in conjunction with dendritic cells enhances antiviral CD8+ CTL responses *in vitro*. J. Clin. Investig., *98:* 715–722, 1996.

20. Guerder, S., Carding, S. R., and Flavell, R. A. B7 costimulation is necessary for the activation of the lytic function in cytotoxic T lymphocyte precursors. J. Immunol., *155:* 5167–5174, 1995.

21. Visseren, M. J. W., van Elsas, A., van der Voort, E. I. H., Ressing, M. E., Kast, W. M., Schrier, P. I., and Melief, J. M. ACTL specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells. J. Immunol., *154:* 3991–3998, 1995.

22. Bakker, A. B. H., Marland, G., de Boer, A. J., Huijbens, R. J. F., Danen, E. H. J., Adema, G. J., and Figdor, C. G. Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells *in vivo*. Cancer Res., *55:* 5330–5334, 1995.

23. Perez-Diez, A., Butterfield, L. H., Li, L., Chakraborty, N. G., Economou, J. S., and Mukherji, B. Generation of $CD8^+$ and $CD4^+$ T-cell response to dendritic cells genetically engineered to express the *MART-1/Melan-A* gene. Cancer Res., *58:* 5305–5309, 1998.

24. Butterfield, L. H., Jilani, S. M., Chakraborty, N. G., Bui, L. A., Ribas, A., Dissette, V. B., Lau, R., Gamradt, S. C., Glaspy, J. A., McBride, W. H., Mukherji, B., and Economou, J. S. Generation of melanoma-specific cytotoxic T lymphocytes by dendritic cells transduced with a MART-1 adenovirus. J. Immunol., *161:* 5607–5613, 1998.

25. Zajac, P., Oertli, D., Spagnoli, G. C., Noppen, C., Schaefer, C., Heberer, M., and Marti, W. R. Generation of tumoricidal cytotoxic T lymphocytes from healthy donors after in stimulation with a replication into competent vaccinia virus encoding MART-1/Melan-A 27–35 epitope. Int. J. Cancer, *71:* 491–496, 1997.

26. Zajac, P., Schutz, A., Oertli, D., Noppen, C., Schaefer, C., Heberer, M., Spagnoli, G. C., and Marti, W. R. Enhanced generation of cytotoxic T lymphocytes using recombinant vaccinia virus expressing human tumor-associated antigens and B7 costimulatory molecules. Cancer Res., *58:* 4567–4571, 1998.

27. Chen, Q., Jackson, H., Gibbs, P., Davis, I. D., Trapani, J., and Cebon, J. Spontaneous T cell responses to melanoma differentiation antigens from melanoma patients and healthy subjects. Cancer Immunol. Immunother., *47:* 191–197, 1998.

28. Chikamatsu, K., Nakano, K., Storkus, W. J., Appella, E., Lotze, M. T., Whiteside, T. L., and DeLeo, A. B. Generation of anti-p53 cytotoxic T lymphocytes from human peripheral blood using autologous dendritic cells. Clin. Cancer Res., *5:* 1281–1288, 1999.

29. Jager, E., Ringhoffer, M., Arand, M., Karbach, J., Jager, D., Ilsemann, C., Hagedorn, M., Oesch, F., and Knuth, A. Cytolytic T cell reactivity against melanoma-associated differentiation antigens in peripheral blood of melanoma patients and healthy individuals. Melanoma Res., *6:* 419–425, 1996.

30. Wasserman, K., Corsi, M. M., Szekely, L., Kono, K., Maes, H. H., and Kiessling, R. Conversion of *in vitro* cultured human monocytes into effective presenters of an HER2/neu-encoded CTL peptide epitope. Scand. J. Immunol., *45:* 678–682, 1996.

31. Ferlazzo, G., Wesa, A., Wei, W. Z., and Galy, A. Dendritic cells generated either from CD34+ progenitor cells or from monocytes differ in their ability to activate antigen-specific CD8+ T cells. J. Immunol., *163:* 3597–3604, 1999.

32. Rongcun, Y., Salazar-Onfray, F., Charo, J., Malmberg, K. J., Evrin, K., Maes, H., Kono, K., Hising, C., Petersson, M., Larsson, O., Lan, L., Appella, E., Sette, A., Celis, E., and Kiessling, R. Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. J. Immunol., *163:* 1037–1044, 1999.

33. Peoples, G. E., Goedegebuure, P. S., Smith, R., Linehan, D. C., Yoshino, I., and Eberlein, T. J. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc. Natl. Acad. Sci. USA, *92:* 432–436, 1995.

34. Brossart, P., Stuhler, G., Flad, T., Stevanovic, S., Rammensee, H. G., Kanz, L., and Brugger, W. HER-2/neu-derived peptides are tumor-associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by *in vitro* induced specific cytotoxic T lymphocytes. Cancer Res., *58:* 732–736, 1998.

35. Ostankovitch, M., Le Gal, F. A., Connan, F., Chassin, D., Choppin, J., and Guillet, J. G. Generation of Melan-A/MART-1-specific CD8+ cytotoxic T lymphocytes from human naïve precursors: helper effect requirement for efficient primary cytotoxic T lymphocyte induction *in vivo*. Intl. J. Cancer, *72:* 987–994, 1997.

36. Lee, T. V., Anderson, B. W., Peoples, G. E., Castilleja, A., Murray, J. L., Gershenson, D. M., and Ioannides, C. G. Identification of activated tumor antigen-reactive CD8+ cells in healthy individuals. Oncol. Rep., *7:* 455–466, 2000.

37. Maeurer, M. J., Chan, H. W., Karback, J., Salter, R. D., Knuth, A., Lotze, M. T., and Storkus, W. J. Amino acid substitutions at position 97 in HLA-A2 segregate cytolysis from cytokine release in MART-1/Melan-A peptide AAGIGILTV-specific cytotoxic T lymphocytes. Eur. J. Immunol., *26:* 2613–2623, 1996.

38. Goldrath, A. W., and Bevan, M. J. Selecting and maintaining a diverse T-cell repertoire. Nature (Lond.), *402:* 255–262, 1999.

39. Lalvani, A. R., Brookes, S., Hambelton, J. J., Britton, A. V. S., Hill, A., and McMichael, A. J. Rapid effector function in CD8+ memory T cells. J. Exp. Med., *186:* 859–865, 1997.

40. Clay, T. M., Custer, M. C., McKee, M. D., Parkhurst, M., Robbins, P. R., Kerstann, K., Wunderlich, J., Rosenberg, S. A., and Nishimura, M. I. Changes in the fine specificity of gp100(209–217)-reactive T cells in patients following vaccination with a peptide modified at an HLA-A2.1 anchor residue. J. Immunol., *162:* 1749–1755, 1999.

41. Lee, K-H., Panelli, M. C., Kim, C. J., Riker, A. I., Betinoti, M. P., Roden, M. M., Fetsch, P., Abati, A., Rosenberg, S. A., and Marincola, F. M. Functional dissociation between local and systemic immune response during anti-melanoma peptide vaccination. J. Immunol., *161:* 4183–4194, 1998.

42. Germain, R. N., and Stefanova, I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. Annu. Rev. Immunol, *17:* 467–522, 1999.

balt6/im-jimm/im-jimm/im1902/im4638-02a | smithl | S=5 | 8/16/02 | 12:48 | Art: 4638 | Input-AGW

Induction of Tumor-Reactive CTL by C-Side Chain Variants of the CTL Epitope HER-2/neu Protooncogene (369-377) Selected by Molecular Modeling of the Peptide: HLA-A2 Complex[1]

Agapito Castilleja,* Darrick Carter,[¶] Clay L. Efferson,* Nancy E. Ward,[†] Kouichiro Kawano,* Bryan Fisk,[2]* Andrzej P. Kudelka,[‡] David M. Gershenson,* James L. Murray,*[*] Catherine A. O'Brian,[†] and Constantin G. Ioannides[3]*[§]

To design side chain variants for modulation of immunogenicity, we modeled the complex of the HLA-A2 molecule with an immunodominant peptide, E75, from the HER-2/neu protooncogene protein recognized by CTL. We identified the side chain orientation of E75. We modified E75 at the central Ser[5] (E75 wild-type), which points upward, by removing successively the HO (variant S5A) and the CH2-OH (variant S5G). Replacement of the OH with an aminopropyl (CH2)3-NH3 (variant S5K) maintained a similar upward orientation of the side chain. S5A and S5G were stronger stimulators while S5K was a weaker stimulator than E75 for induction of lytic function, indicating that the OH group and its extension hindered TCR activation. S5K-CTL survived longer than did CTL induced by E75 and the variants S5A and S5G, which became apoptotic after restimulation with the inducer. S5K-CTL also recognized E75 endogenously presented by the tumor by IFN-γ production and specific cytolysis. AQ: A **S5K-CTL expanded at stimulation with E75 or with E75 plus agonistic anti-Fas mAb. Compared with S5K-CTL that had been restimulated with the inducer S5K, S5K-CTL stimulated with wild-type E75 expressed higher levels of E75[+] TCR and BCL-2. Activation of human tumor-reactive CTL by weaker agonists than the nominal Ag, followed by expansion with the nominal Ag, is a novel approach to antitumor CTL development. Fine tuning of activation of tumor-reactive CTL by weak agonists, designed by molecular modeling, may circumvent cell death or tolerization induced by tumor Ag, and thus, may provide a novel approach to the rational design of human cancer vaccines.** *The Journal of Immunology,* **2002, 169: 0000–0000.**

Induction of tumor-reactive CTL by vaccination is a promising approach to cancer therapy. Because tumor Ags are weak immunogens, their immunogenicity must be enhanced if the vaccine is expected to induce antitumor CTL-effector responses. Enhancement of immunogenicity is determined by the ability of the modified agonistic tumor Ag to induce higher levels of effector responses than does the wild-type epitope itself. The higher sensitivity of the agonist-induced CTL for the wild-type Ag is illustrated by higher levels of cytokine secretion and higher levels of cytolysis at the encounter with the tumor Ag or the tumor itself. Strong agonistic immunogens are generally designed by one of two general approaches: 1) to modify immunogens so that they bind the HLA-A, B, C-presenting molecule with higher affinity than their corresponding wild-type counterparts; or 2) to modify the TCR contact site so that agonistic variants of the tumor Ag can enhance the responses of T cells by their TCR contacts (reviewed in Ref. 1). The first approach has been used successfully for normally low-affinity binding HLA-A2 peptides such as MelA, C85, and GP2 (2–7). The second approach is currently used for higher affinity MHC-I-binding peptides. The rationale of the second approach is to replace residues in the Ag that contribute less to the peptide binding affinity for MHC-I and are less likely to contact the TCR with other residues which by their size can create novel contacts for the TCR (1, 8–9).

Mutation of naturally occurring peptides recognized with high affinity at their TCR contacting residues usually results in less potent ligands (10). Thus, mutation of a CTL epitope can lead to a partial agonist or an antagonist. In this regard, one approach for producing stronger agonists has been to modify the surface conformation of the MHC molecule by using buried peptide side chains (11) or buried phenolic groups (12). This also augmented the number of TCR specificities that responded to a single peptide determinant (11, 12). A novel approach to change the MHC affinity for TCR is to modify only the side chains of the amino acids that can contact the TCR. This approach requires identification of such side chains and selective use of modifications so as to enhance tumor Ag stimulation ability while avoiding CTL death from overstimulation. Because only the wild-type Ag is presented in vivo, a central requirement to be fulfilled by side chain modifications of the peptide is that the cells that are activated by the variant must survive at encounter with the wild-type Ag. This means that the wild-type Ag should induce the same or better protection from death by apoptosis in CTL that have been induced by the variant than the variant itself.

Modulation of immunogenicity in this way requires identifying the peptide-MHC-1 complex (pMHC-1) structure, the side chains

Departments of *Gynecologic Oncology, [†]Cancer Biology, [‡]Gynecologic Medical Oncology, [§]Immunology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030; and [¶]Corixa Corporation, Seattle, WA 98104

Received for publication April 16, 2002. Accepted for publication July 29, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

AQ: I [1] This work was supported in part by Grants DAMD-17-97-1-7098 and 01-1-0299. Peptide synthesis was supported in part by the M. D. Anderson Cancer Center Core Grant CA 16672.

[2] Current address: Department of Internal Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814.

[3] Address correspondence and reprint requests to Dr. Constantin G. Ioannides, Department of Gynecologic Oncology, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Room T4.3891, Box 440, Houston, TX 77030. E-mail address: cioannid@mdanderson.org

0022-1767/02/$02.00

pointing upwards in the central peptide area, and using as replacements peptides whose side chains have similar degrees of freedom for flexible orientation at the central position so that they differ in their biological potency. The pMHC-I structure can be modeled by using as a "search model" the crystal structure of another peptide that has structural similarities with the pMHC-I (13, 14). Identification of the positioning of the side chains of the residues in the central area at amino acid positions 4–7 allows changes to be focused in the area that complements the TCR combining site. This area was recently identified as the functional "hot spot" that allows TCR to finely discriminate among similar ligands (10). A side chain in the central area pointing upwards (toward the TCR) can achieve more extensive contact with the TCR than others. This contact is provided by an increase in van der Waals forces from the hydrophobic side chains, or by an increase/decrease in hydrogen bonds by OH groups, or by an increase/decrease in charged interactions. Whether the side chain extension correlates with increased immunogenicity remains unknown.

To address these questions, we examined the binding of the HER-2/neu protooncogene (HER-2), CTL epitope E75 (369-377) to HLA-A2 at the atomic level. Molecular models of the E75-HLA-A2 complex indicated that the side chain of the central Ser^5 (S373) points upward. Thus, the OH group can either enhance binding at the TCR via a hydrogen bond, or sterically hinder the interaction with the TCR by decreasing the affinity of the TCR for the pMHC-I. If the first hypothesis is true, then removal of the OH group should decrease the affinity of binding by the TCR and decrease signaling, hence variants in which the central Ser is replaced by Ala or Gly should be less immunogenic than wild-type E75. If the second hypothesis is true, then Ala/Gly variants should be more immunogenic than the wild-type E75. To address the requirement that variant-induced CTLs survive their encounter with the wild-type Ag, we created another variant reasoning that stimulation with that variant should protect responding cells from death by overstimulation. This variant should stimulate some of the effector functions weaker than E75, and E75 should activate the variant-induced effectors. The only alternatives that would not disturb the peptide bond were positively and negatively charged side chains. Because the negatively charged amino acids Glu and Asp have bulky carboxyl groups, we replaced Ser^5 with the positively charged Lys^5 (variant S5K). The aminopropyl group of Lys extends farther and has a greater flexibility than the acetyl group of the Glu.

Priming with variants S5A and S5G enhanced the induction of IFN-γ and E75-specific cytolysis of CTL from two donors known to respond to E75, but the responders died faster than did the cells that had been stimulated by E75. In contrast, variant S5K induced higher levels of IFN-γ, but not of CTL activity against E75 than the E75-induced CTL (E75-CTL). In a "weak responder" to E75, S5K-induced CTL (S5K-CTL) recognized E75 with lower affinity than did E75-induced CTL. S5K-CTL survived longer than the E75-CTL, which became apoptotic at restimulation with E75. Of interest, restimulation with E75 resulted in better protection from apoptosis in the S5K-CTL than did restimulation with S5K. This protection was paralleled by higher Bcl-x_L to Bad ratios and higher Bcl-2 levels than the ones induced by S5K. Thus, the side chain variants that were less activating than the wild-type Ag induced specific CTL for the E75 expressed on tumors. Such CTL were then expanded by E75, indicating that the nominal Ag or stronger agonistic variants can use priming with weak agonists to bypass induction of apoptosis.

Materials and Methods

Cells, Abs, and cytokines

HLA-$A2^+$ and PBMC were obtained from completely HLA-typed healthy volunteers. T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (15–17). mAb to CD3, CD4, CD8 (Ortho Diagnostics, Rantory, NJ). CD13 and CD14 (Caltag Laboratories, San Francisco, CA), and HLA-A2 (clone BB7.2; American Type Culture Collection, Manassas, VA) were either unconjugated or conjugated with FITC or PE. Ag expression by dendritic cells (DCs)[4] and T cells was determined by FACS analysis using a flow cytometer (EPICS-Profile Analyzer; Coulter Electronics, Hialeah, FL). GM-CSF of specific activity (1.25×10^7 CFU/250 mg) was from Immunex, Seattle, WA; TNF-α of specific activity (2.5×10^7 U/mg) was from Cetus (Emeryville, CA); IL-4 of specific activity (5×10^6 IU/mg) was from Biosource International (Camarillo, CA); IL-2 of specific activity (18×10^6 IU/mg) was from Cetus; IL-12 of specific activity (5×10^6 U/mg) was a kind gift from Dr. S. Wolf (Department of Immunology, Genetics Institute, Cambridge, MA). The anti-human-Fas mAb CH11 was purchased from Upstate Biotechnology (Lake Placid, NY). mAb to actin, Bcl-2, Bcl-x_L, and Bad were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other specific mAb and isotype controls were obtained from BD PharMingen (San Diego, CA).

Synthetic peptides

Peptides used were E75 (HER-2: 369-377) and its mutated analogs (Table I). To facilitate presentation, E75 variants mutated at Ser^5 are abbreviated based on the position and the substitution. For example, the variant in which serine was replaced by alanine is S5A; the variant in which serine was replaced with glycine is S5G. A7.3 in which the alanine side chain was extended with two methylene groups was obtained by replacement of Ala with Norleucine (linear side chain). F8-1 was obtained by replacing of Phe^8 with isophenylalanine (1 CH2) deletion. All peptides were prepared by the Synthetic Antigen Laboratory of M. D. Anderson Cancer Center (Houston, TX) and purified by HPLC. The purity of the peptides ranged from 95–97%. Peptides were dissolved in PBS and stored frozen at −20°C in aliquots of 2 mg/ml.

Molecular modeling of the peptide: HLA-A2 complex

The coordinates of the native HLA-A2 structure (14, 18, 19) were downloaded from the Brookhaven protein database (ID number: 3HLA). This file was used as a template for manipulations with the Swiss Model (20) program available through the Expasy web site. The Tax peptide bound to the HLA-A2 (21) was mutated manually to yield the bound E75 peptide and the Ala^5, Gly^5, and Lys^5 variants. Each new structure was submitted for energy minimization with the GROMOS96 implementation of the Swiss-PdbViewer. Solvent-accessible surface area was calculated with the GETAREA1.1 online program with the default probe radius, set at 1.4 Å.

T cell stimulation by peptide-pulsed DC

DCs generated from peripheral blood were plated at 1.2×10^5 cell/well in 24-well culture plates and pulsed with peptides at 50 μg/ml in serum-free medium for 2 h before the addition of responders, as described (15, 16). E75-induced and S5K-induced CTL lines were maintained by periodic stimulation with peptide pulsed on DCs, followed by expansion in the presence of irradiated feeder cells and PHA. The number of cells expressing a TCR that was specific for HLA-A2 bound to the E75 peptide (E75-TCR^+ cells) was performed using E75 dimers (dE75) prepared as described in the manufacturer's instructions. Empty HLA-A2:IgG dimers were obtained from BD Pharmingen. Control without peptide dimers not pulsed with peptide (NP) were prepared in parallel and tested in the same experiment. Positive control influenza matrix peptide M1 (58–66) dimers (dM1) were prepared simultaneously and used in the same experiment. For analysis, cells were incubated in parallel with dNP, and dE75 followed by PE-conjugated anti-mouse IgG1. Intracellular expression of Bcl-2 was determined, following manufacturer's instructions using FITC-conjugated Bcl-2, Ab, and a matched FITC-conjugated isotype control.

CTL and cytokine assays

Recognition by CTL of peptides used as immunogens was performed as described (17). Recognition of E75 and of its variants was considered specific when the percent specific lysis of T2 cells pulsed with E75 minus

[4] Abbreviations used in this paper: pMHC-I, peptide-MHC-I complex; HER-2, HER-2/neu protooncogene; DC, dendritic cell; NP, not pulsed with peptide; FW, forward scatter; d, dimer.

Table I. *HLA-A2 binding stability by E75 and its variants*[a]

Code	Sequence	Binding Stability	Ligation[b] Strength	Change
E75	KIFGSLAFL	482	28	Wild type
K1G	GIFGSLAFL	138	28	Positive charge→neutral
S5A	KIFGALAFL	482	28	OH→nonpolar aliphatic
S5G	KIFGGLAFL	483	30	OH→neutral
S5K	KIFGKLAFL	482	29	OH→positive charge
F8K	KIFSGSLAKL	88	30	Aromatic to (+) charged
F8Y	KIFGSLAYL	482	28	OH in aromatic residue
F8D	KIFGSLADL	236	28	Aromatic to (−) charged
A7.3	K1FGSL (NLeu)FL	nd[c]	nd	2 CH2 extension of Ala^7
F8-1	K1FGSLA (Iso-Phe)L	nd	nd	1 CH2 deletion of Phe^8

[a] The binding stability is an estimate of half time of dissociation (in minutes) from HLA-A2 of peptides of the sequence listed above. The theoretical half-life of dissociation was calculated using Parker's algorithm (27) available at http://bimas.dcrt.ig.gov/molbiol/hla-bind.

[b] The ligation strength was calculated using the SYFPEITHI program (28). The experimentally determined mean channel fluorescence values for HLA-A2 expression on T2 cells after incubation with peptides and staining with MA.2.1 mAb were: NP = 90, E75 = 305, S5G = 295, S5A = 290, S5K = 285, K1G = 240, and F8Y = 305.

[c] nd, not done.

the SD was higher by at least 5% than the percentage of specific lysis of T2 cells that had been pulsed with peptide plus the SD, as described (22). A significant increase/decrease in CTL activity was defined as an increase/decrease of >20% in the lysis of T2 cells pulsed with peptide by variant-induced CTL compared with wild-type E75-induced CTL. Similarly, a significant increase in IFN-γ induction was defined as an increase of >20% in IFN-γ levels after stimulation with the variant vs after stimulation with the wild-type E75. The 20% value was chosen as a cut-off for significant increase based on the assumption that if a 2-fold increase of the minimum 5% increase (defined above) is 10%, then an increase >10% should be significant if it equals at least 20%. Equal numbers of viable effectors were used in all assays. IL-2, IL-4, and IFN-γ were detected using cytokine ELISA kits (Biosource International or R&D Systems, Minneapolis, MN) with a sensitivity of 4–7 pg/ml (15).

Apoptosis assays

E75- and S5K-CTL lines were activated by autologous DCs pulsed with various concentrations of E75 or S5K in the presence or absence of 100 μg/ml of CH11. For anti-CD3-mediated apoptosis, OKT3 mAb was absorbed on wells of 96-well plates overnight before addition of lymphocytes (23). For day 1 apoptosis assays, IL-2 was not added to the cultures. For day 4 apoptosis assays, IL-2 (300 IU/ml) was added to the cultures at 24 and 72 h after stimulation with DC-pulsed peptides. Detection of Fas-mediated apoptosis was performed in the presence or absence of the agonistic mAb CH11 (anti-Fas mAb) as described (23). Cells were labeled by incubation in PBS containing 0.1% Triton X-100 and 50 μg/ml propidium iodide, and the DNA content was determined by using flow cytometry.

Western analysis

A total of 2×10^6 S5K-$CD8^+$ cells were stimulated for 96 h with E75, S5K, A7.3, or F8-1 peptides pulsed on DCs at a final concentration of 25 μg/ml. Additional controls included cells that were stimulated with T2 that had not been pulsed with peptide, or S5K cells that were not stimulated or cells that were stimulated with PHA. A total of 20 μg of protein from supernatants from 10,000 g of postnuclear detergent lysates were separated on a 12% SDS-PAGE gel and immunoblotted as described (24). Membranes were probed with monoclonal anti-actin, anti-Bcl-2 (1:500), anti-Bad (1:500), or anti-Bcl-x_L (1:500) in 1% BSA-TBS containing 0.1% Tween 20 for 2 h at 25°C, and probed with peroxidase-linked sheep anti-mouse Ig (1:1000) in 1% BSA-TBS containing 0.1% Tween 20. Immunoreactive bands were detected by ECL as described (24).

Results

Generation of E75 variants directed by molecular modeling

The rationale for this approach was to identify amino acids in E75 permissive to replacement that would be substituted without abolishing the objects of the variant peptide to induce CTL responses. Substitutions in side chains that maintain the overall conformation of the peptide backbone in the HLA-2 are more likely to lead to cross-reactive Ag for wild-type Ag-specific CTL than are substitutions that change the peptide backbone conformation. We modeled the E75-HLA-A2 complex by replacing the human T cell leukemia virus-1 peptide Tax with E75. The Tax peptide (25, 26) shows the highest structural similarity with E75 of the models available in the databases. The Tax sequence *LLFGYPVYV* is similar to that of E75:*KIFG* SL *AFL* with respect to the position of aromatic residues in P3 and P8 and the aliphatic side chain extensions in the first four and the last three amino acids (only K1 and F8 differ by an NH3 and an OH group extension). The major differences rest in the central area P5 P6:YP vs SL. One Tax analog, P6A, shows even more similarity with E75 YA vs SL, with Ala and Leu differing only in the propyl side chain. This comparison allowed identification of the side chains that point upwards or sideways and will be more likely to contact TCR. The results show that the side chains of Lys^1, Ser^5, and Phe^8 point out of the binding pocket of the MHC (Fig. 1*A*). The side chains of Phe^3, Leu^6, and Ala^7 point toward the helical "walls" of the pocket (Fig. 1*A*). The models of the TCR-pMHC-I (HLA-A2) interaction predict that of the side chains pointing away from the MHC, Ser^5, Leu^6, and Ala^7 are most likely to contact the CDR3 (Vα + Vβ) region. We focused on Ser^5 because the change induced by the removal of the hydroxyl group was likely to have the strongest effects.

Ser was substituted with Ala, Gly, and Lys. These substitutions removed an HO-group (Ala), a HO-CH2-group (Gly), or replaced the OH group with the aminopropyl (CH2-CH2-CH2-NH3) group. The position of the OH suggests that it is less involved in interactions with the HLA-A2 (Fig. 1*A*). No significant changes of the MHC molecule were necessary to accommodate these modifications (Fig. 1, *B–D*). Ser^5 is preceded by Gly^4, which because it does not have a side chain, is very flexible and may allow small accommodations in the model. The positions of Phe^3 and Lys^1 that precede the Ser^5 seem to be unchanged among the four models. These results indicate that Ser^5 is in a good structural position to allow side chain replacements in the antigenic peptide that can modify its interactions with TCR. S5A, S5G, and S5K bound to HLA-A2 with similar affinity as did E75 (Table I). In T2-stabilization assays, S5A, S5G, and S5K showed similar stabilizing ability for HLA-A2 as determined with mAb MA2.1 (Table I, legend), and similar scores for times of dissociation and ligation strengths (Table I) with those of E75 as determined using the HLA-peptide binding prediction (27) and SYFPEITHI programs (28).

Increased IFN-γ-inducing and E75-specific CTL-inducing ability of the E75-variants S5A and S5G

To address whether modification of the E75 side chain by deletion or extension would increase or decrease the ability of the modified Ag to stimulate CTL induction and survival, we tested several healthy donors known from previous studies to produce E75-specific CTL at priming ("strong responders", donors 1 and 2) or exhibit weak CTL activity after several repeated stimulations (weak responders, donor 3). PBMC were stimulated in parallel with autologous DCs pulsed with E75 variants. Donor 1 responded with higher levels of IFN-γ at priming with variants S5K, S5G, and S5A, and lower levels of IFN-γ at priming with control variants F8Y and F8K than at priming with E75 (Fig. 2, *A* and *B*). CTL induced by priming with E75 recognized E75 better than did CTL induced by S5K, F8Y, or F8K, whereas CTL induced by S5G and S5A recognized E75 better than CTL induced by E75. S5A and S5G induced both higher levels of IFN-γ and higher cytolytic activity than did E75. Thus, removal of the OH group correlated with

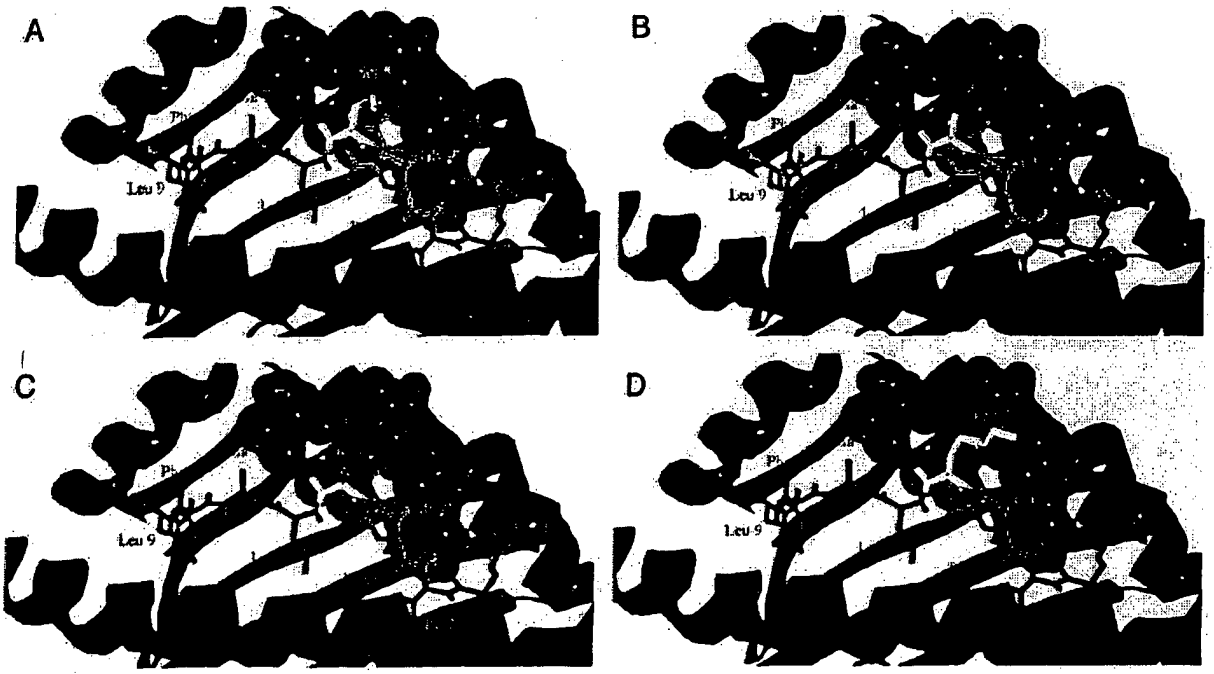


FIGURE 1. *A*, Molecular model of the E75 peptide bound to the MHC. The MHC-peptide complex was modeled as described in *Materials and Methods*, and rendered using the POV-Ray implementation of the Swiss PdbViewer. Residues within 6 A of Ser[5] are displayed with their van der Waals radii in red. Ser[5] is in yellow, while the other E75 peptide residues are in green. The model is presented rotated with an angle of 180° to facilitate distinction of the side chains pointing upwards. *Top*, ~~anti~~-2 domain; *bottom*, ~~anti~~-1 domain of HLA-A2. HLA-2 is depicted in blue. *B*, Similar view of the complex for the Ser[5] to Ala[5] model of the peptide-MHC complex. *C*, The Ser[5] to Gly[5] model. *D*, The Ser to Lys[5] model. The same orientation is used as in all the molecules above.

alpha alpha

higher IFN-γ induction and higher lytic activity against E75. CTL induced by S5K secreted higher levels of IFN-γ, but their recognition of E75 was weaker. Thus, replacement of OH group with aminopropyl group had more selective effect than removal of the OH group. Extension of these results with cells from donor 2 revealed that all at the E75 variants induced higher levels of IFN-γ at priming than did E75: S5K by 36%, S5A by 100%, and S5G by 64% (Fig. 2*C*). Significantly higher levels of IFN-γ were detected 96 h after stimulation with each variant in response to the highest dose (25 μg) of exogenously pulsed peptide in the presence of IL-2 for 2 days. Significant differences in IFN-γ induction were not observed when E75 or its variants were used at 1.0 or 5.0 μg/ml at 48 or 72 h. The E75-specific lytic activity of CTL induced by S5A was significantly higher than the lytic activity of CTL induced by E75 (Fig. 2*D*). The increase in lytic activity by S5A paralleled the increase in IFN-γ in response to S5A. Recognition of E75 by S5K-CTL was lower than the recognition by E75-CTL. CTL induced by the E75, S5K-CTL, and S5A-CTL all recognized the indicator SKOV3.A2 tumor. To determine whether E75-specific tumor-lytic CTLs were present in the variant-induced CTL, we performed cold-target inhibition of tumor lysis. Tumor lysis by S5K-CTL was inhibited less by T2-E75 than lysis by E75-CTL (Fig. 2*E*). This confirmation that S5A can induce both higher IFN-γ and higher lytic activity against E75 suggested that the OH group of Ser[5] hindered the TCR interaction with peptide-HLA-A2 and that removal of the OH group allowed a stronger TCR activation. However, at restimulation, the number of cells stimulated by S5A and S5G dropped faster than the number of cells that had been stimulated by E75. Cells stimulated by S5K survived longer than E75-stimulated cells (Fig. 2*F*), suggesting that the stimulus from the (CH2)3-NH3 was more effective than stimuli from the CH3 or the CH2-OH in maintaining the survival of responders.

Stimulation with S5K enhanced survival of responding T cells

Cancer patients are weak responders to E75 and require repeated stimulation for CTL induction. To clarify the differences between E75 and S5K in the induction of cytolysis, we tested T cells from donor 3 for whom several stimulations with E75 were required to induce detectable CTL activity, but responded with IFN-γ secretion at priming (16). S5K and E75 induced similar levels of IFN-γ at priming and at restimulation (Fig. 3*A*). The kinetics of induction of E75-specific CTL in relation to the number of stimulations is shown in Fig. 3*B*. E75 again induced higher E75-specific lytic activity than did S5K. Like donor 2, E75-stimulated cells from donor 3 declined in number after the third stimulation with Ag more than the S5K-stimulated cells (Fig. 3*C*). These results showed that S5K induced better survival in responders than E75. These results were confirmed in subsequent stimulation experiments. In parallel experiments, priming with E75 induced lower levels of Bcl-2 in CD8$^+$ cells than did priming with S5K. There were only small differences in Fas ligand, Fas, and IL-2Rα expression between E75-stimulated and S5K-stimulated donor 3 CD8$^+$ cells (A. Castilleja et al., unpublished observations).

F3

AQ: C

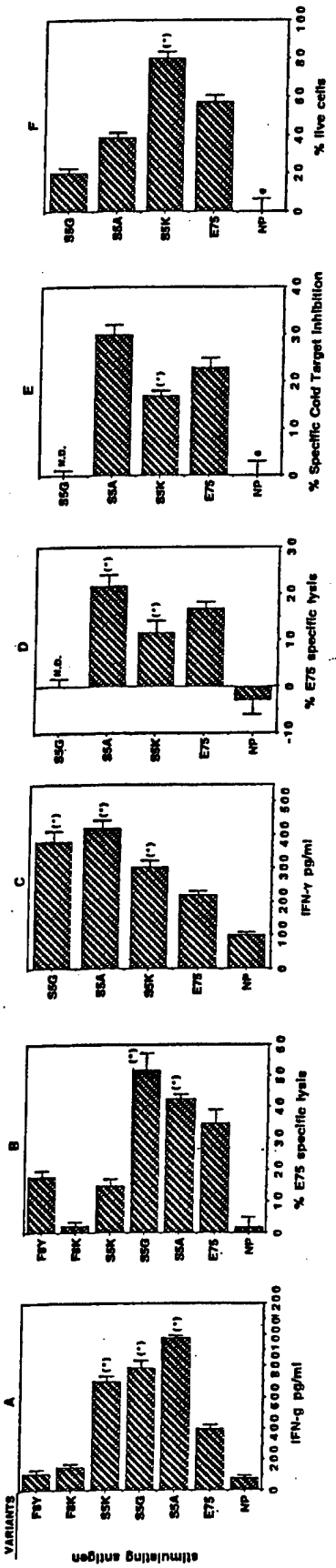
AQ: D

and C.G. Ioannides

S5K-induced CTL recognized E75 with lower affinity than E75-induced CTL

Weaker recognition of E75 by the S5K-CTL raised the question of whether S5K induced smaller numbers of CTL than E75, or whether the CTL induced by S5K had lower affinity for E75 than for S5K. To address the recognition of variant-induced CTL, we tested their ability to recognize E75 and the inducing variant in parallel. S5A-CTL (donor 1) recognized S5K weaker than S5A (24% decrease), suggesting that extension of the CH2 side chain in

F4



AQ: E

position 5 with OH and (CH2)3-NH3 groups, respectively, hindered TCR recognition. Similarly, donor 3 S5K-CTL recognized E75 weaker than they recognized S5K (Fig. 4*A*). To verify that S5K is recognized with lower affinity than E75 by donor 3 E75-CTL, we performed concentration-dependent lysis. E75-CTL recognized S5K with lower affinity than E75. S5K recognition was close to recognition of E75 (32 vs 41%) only at high concentrations (50 μg/ml; Fig. 4*B*). Similarly, S5K-CTL recognized E75 with lower affinity than S5K (Fig. 4*C*). These results demonstrated that the OH and aminopropyl groups selectively modulated the affinity of recognition. To address whether E75-specific CTL were present in smaller numbers in S5K-CTL, we tested recognition of E75 at the same concentration (10 μg/ml) at four E:T ratios (10, 20, 30, 40). Even at the highest E:T ratio of 40:1, S5K-CTL recognized E75 (25.4% lysis) to a significantly lesser extent than did E75-CTL at an E:T ratio of 10:1 (48.2% lysis).

S5K-CTL recognize endogenously presented E75

Because S5K-CTL survived longer than E75-CTL, this raised the possibility that S5K could be used to induce CTL-recognizing tumors. To determine whether S5K-CTL recognized endogenous E75 in cytolysis assays, we performed cold-target inhibition of tumor lysis. T2-E75 inhibited lysis of freshly isolated ovarian tumor OVA-16 (HLA-A2$^+$, HER-2^{high}) by 21% in an 8-h CTL assay, and by 45% in a 16-h assay (Fig. 5, *A* and *B*). Similar inhibition (38%) was observed against SKOV3.A2 in a 16-h assay (data not shown). These results indicated that S5K-CTL recognized the endogenously presented E75 and ovarian tumors overexpressing HER-2. The levels of inhibition of lysis indicative of specific recognition were similar to those levels observed with donor 2, E75-CTL, and S5K-CTL (Fig. 2*E*). We also tested S5K-CTL ability to secrete IFN-γ at an encounter with the ovarian tumor SKOV3.A2 and its HLA-A2$^-$ counterpart SKOV3. This was necessary because the tumor and responding lymphocytes shared HLA-A3. S5K cells secreted high levels of IFN-γ within 20 h, when IL-12 was used as costimulator (Fig. 5*C*). IFN-γ was induced even in the absence of IL-12, but at lower levels. mAb inhibition experiments indicated that IFN-γ secretion was associated with recognition of HLA-A2. (data not shown). This indicated that present among the S5K-induced CTL was a subpopulation of cells that recognized endogenously presented E75 by cytolysis and IFN-γ secretion.

FIGURE 2. Induction of effector functions in donor 1 (*A* and *B*) and donor 2 (*C–E*) at priming with the wild-type CTL epitope E75 and its variants. *A* and *C*, IFN-γ; *B*, *D*, and *E*, Cytolysis. *A* and *C*, IFN-γ was determined from supernatants collected from the same cultures which were used on day 8 for CTL assays. *B*, *D*, and *E*, Equal numbers of effectors from each culture were tested in the same experiment. Results indicate the percentage of E75-specific lysis obtained by subtracting the specific lysis of T2 cells not pulsed with peptide, from the specific lysis of T2 cells pulsed with 25 μg/ml E75 in the same experiment. The E:T was 20:1. Stimulators were autologous DCs pulsed with 25 μg/ml peptide. NPs indicate control effectors that were stimulated only with autologous DCs which were not pulsed with peptide. *E*, Effectors E75-CTL, S5K-CTL, and S5A-CTL lysed the indicator ovarian tumor SKOV3.A2. Specific cold target inhibition indicates the percentage of inhibition of lysis of SKOV3.2 cells by cold (unlabeled) T2-E75 cells minus inhibition of lysis in the presence of T2-NP cells. S5G-CTL were not used in this experiment because their numbers declined rapidly after restimulation. E:T ratio was 30:1, cold:hot ratio was 10:1. *F*, Percentage of live cells in donor 2 cultures primed and restimulated with each variant 30 days after priming. Note the decrease in live cells in cultures stimulated with S5A or S5G. *, $p < 0.05$.

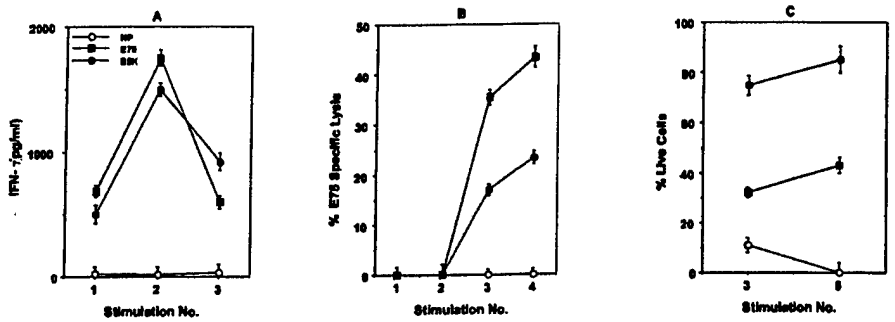


FIGURE 3. *A*, Kinetics of IFN-γ production; *B*, E75-specific CTL induction; and *C*, survival of donor 3 CTL stimulated by E75 and S5K. Experimental details as described in the text and the legend to the Fig. 2. *A*, IFN-γ was determined on day 3 after stimulation with each peptide. The numbers 1, 2, and 3 indicate the number of stimulations. Equal numbers of live cells from E75- and S5K-stimulated cultures were stimulated with autologous DC pulsed with the corresponding peptide. *C*, The number of live cells recovered was determined 1 wk after the third and the fifth stimulations.

Antiapoptotic effects of E75 in S5K-activated CD8⁺ cells

Induction of CTL by the variant S5K raised the question of whether such cells could survive an encounter with E75 since E75 is present in vivo. To address whether E75 can induce CD95-mediated apoptosis, E75-CTL and S5K-CTL were stimulated with E75 and S5K in parallel in the presence of the agonistic Ab CH11. Three days after stimulation with E75, 46% of the E75-CTL had undergone apoptosis, whereas only 15.4% of the S5K-CTL were apoptotic after stimulation with S5K (data not shown). In contrast, when S5K-CTL were stimulated with S5K or E75, cells stimulated with E75 survived longer and may have increased in number as compared with the cells stimulated with S5K. Stimulation of S5K-CTL with 25 or 50 μg/ml E75 for 4 days increased the number of CD8⁺ cells by 26 and 64%, respectively. Stimulation of the same cells with S5K + anti-Fas increased their numbers by 0.93- and 27%, respectively (Fig. 6, *A* and *B*), but no increase in cell number was observed in the absence of CH11. Notably, S5K-CTL continued to respond to S5K with higher levels of IFN-γ, but lower levels of IL-2, than did cells treated with E75 (data not shown).

To address whether E75 and S5K interfered with apoptosis pathways, S5K-CTL were restimulated with E75 or S5K at two different concentrations or remained unstimulated (Group 0, DC only) in the presence of CH11. Apoptosis analysis was performed at 24 and 96 h. Both E75 and S5K inhibited the residual Fas-apoptosis within 24 h and this inhibition was peptide concentration-dependent (Fig. 6*C*). When apoptotic cells were counted on day 4, both peptides were protective, but E75 seemed to be more protective than S5K (Fig. 6*C*, day 4).

To confirm the antiapoptotic effects of E75 and S5K on S5K-CTL, we performed cell cycle analysis. Analysis of cells in the subG$_1$ phase (Fig. 6*D*) showed that 46% of the unstimulated S5K cells became apoptotic. E75 and E75 + CH11 inhibited this apoptosis by 83%. S5K had a slightly lower inhibitory effect (63% inhibition). S5K + CH11 reduced apoptosis by only 24% compared with unstimulated S5K-CTL confirming the results in Fig. 6*B*. The percentage of cells in G$_1$ phase (resting) was similar in both stimulated and control unstimulated cells (50 ± 5%). The percentages of CD8⁺ cells in S phase in cultures stimulated with E75 or S5K were also similar. Of interest, the proportion of cells in the S phase was higher in cultures stimulated by E75 + CH11 than in cultures stimulated with S5K + CH11, suggesting that E75 transmitted a stronger stimulatory signal for division of S5K-CTL than their original inducing Ag. The differences between cells in the G$_{2/M}$ phase were small compared with the unstimulated cells, and they were not considered significant. These results agree with the higher proliferation of S5K-activated CD8⁺ cells in response to E75 than to S5K (Fig. 6, *A* and *B*).

Apoptosis resistance in stimulated T cells at day 4 is mainly due to the intrinsic pathway (29). Because resistance to Fas-induced apoptosis was suggestive of TCR-induced protection, we investigated the effects of E75 and S5K in up-regulation of Bcl-2, Bcl-x$_L$, and Bad. Unstimulated and DC-NP-stimulated CD8⁺ cells from

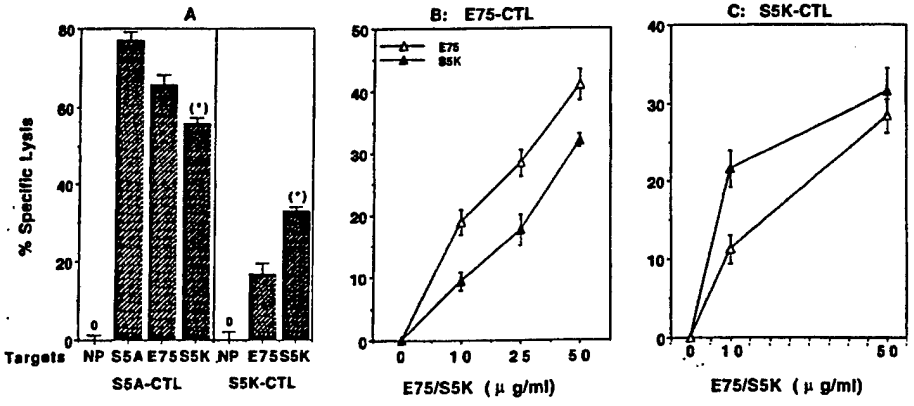


FIGURE 4. Ag specificity of S5A-CTL, S5K-CTL, and E75-CTL. *A*, Donor 1 S5A-CTL recognized S5K less efficiently than S5A. Donor 3 S5K-CTL recognized E75 with lower affinity than S5K. T2 cells were pulsed with E75 and S5K at 10 μg/ml. *B*, Donor 3 E75-CTL recognized S5K with lower affinity than E75. *C*, Donor 3 S5K-CTL recognized E75 with lower affinity than S5K-CTL. Concentration dependent recognition of E75 and S5K in the same experiment. Targets were T2 cells pulsed with the indicated concentrations of peptide. *B* and *C*, Results of a 6-h CTL assay. E:T ratio was 10:1. *, $p < 0.05$.

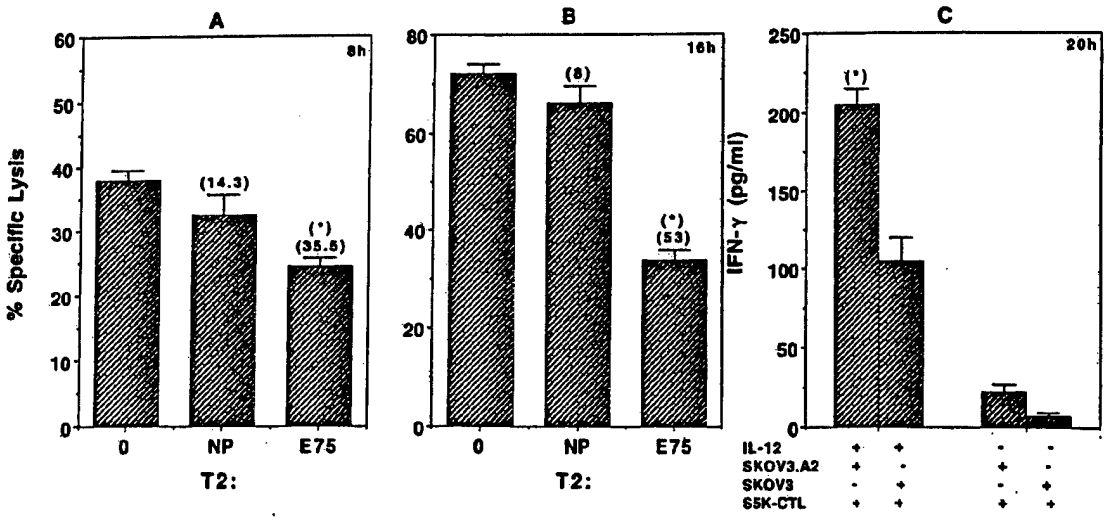


FIGURE 5. S5K-CTL recognized endogenous E75 presented by ovarian tumor cells. *A* and *B*, Cold target inhibition of cytolysis of OVA-16 (HLA-A2, HER-2^{high}). Cold targets were T2 pulsed with E75, using as specificity control T2 which were not pulsed with peptide (T2-NP). Numbers in the parentheses indicate the percentage of inhibition of lysis of S5K-CTL by T2-E75 compared with lysis of tumor in the presence of T2-NP. *, $p < 0.05$. E:T ratio was 10:1; the ratio of cold to hot targets was 1:1. *C*, IFN-γ induction. IL-12 was used at 3 IU (300 pg/ml); the responders to SKOV3. A2 stimulator ratio was 40:1.

S5K-CTL were used as negative controls, while S5K-CTL stimulated with the agonists A7.3 and F8-1 were used as positive controls. E75 induced a higher Bcl-x_L to Bad ratio than S5K. A7.3 and F8-1 variants induced even higher Bcl-x_L to Bad ratios than E75, indicating that their effects were sequence-specific (Fig. 7*A*). S5K was a slightly stronger up-regulator of Bcl-2 than E75. The inhibitory effects of E75 and S5K on Bad up-regulation were similar, although E75 was a slightly stronger inhibitor. These results indicate that E75-mediated protection from CD95-mediated apoptosis of S5K-CTL correlated with down-regulation of proapoptotic family members. The increase in the level of expression of Bcl-2 was considered significant compared with the up-regulation of Bcl-2 induced by a mitogen (PHA) in the same cells for 96 h. This is evident when the Bcl-2 and Bcl-x_L to actin ratios are compared at stimulation with S5K and PHA vs the Bcl-2 and Bcl-x_L to actin ratios in unstimulated cells (Fig. 7*B*). For S5K stimulation, the ratios are 1.72 (Bcl-2) and 1.32 (Bcl-x_L), while for PHA stimulation the ratios are 1.55 (Bcl-2) and 4.37 (Bcl-x_L). The increase in the levels of Bcl-2 and Bcl-x_L at stimulation with PHA is comparable with the increase reported in other studies in the presence of a mitogen, but in the absence of IL-2. Increase in the Bcl-2 levels is in general observed if mitogen-activated T cells are given high doses of IL-2 (30, 31). Thus, activation and expansion of tumor-reactive CTL by the variant S5K allowed better survival of these CTL in response to the wild-type tumor Ag.

To address whether E75 and S5K stimulation affected expansion, TCR expression, and Bcl-2 expression in $E75^+$TCR cells, S5K-CTL were stimulated with T2 cells pulsed with either E75 or S5K or not pulsed with peptide (T2-NP). The number of $E75^+$TCR cells was determined. One week later, to determine whether the affinity of the TCR for E75 was affected by the stimulation, we assessed expression of $E75^+$TCR cells both immediately

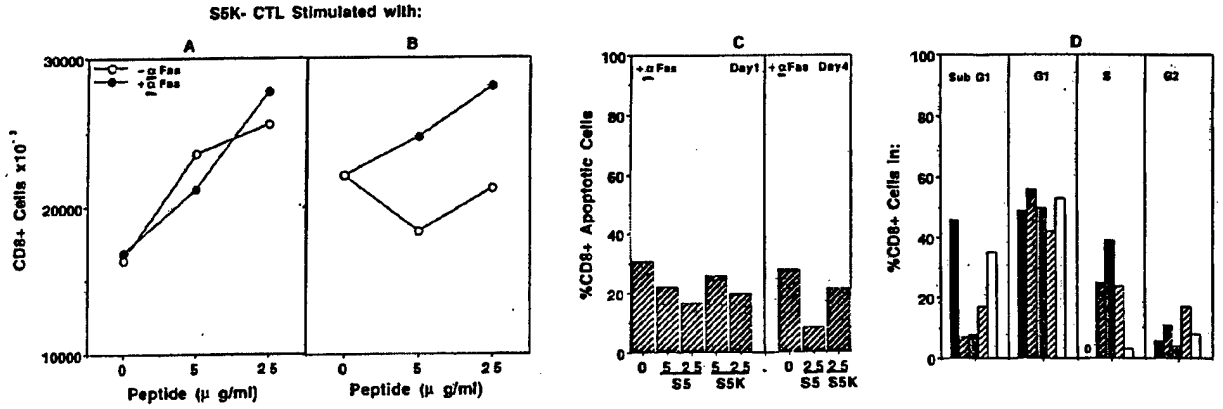


FIGURE 6. Expansion of $CD8^+$ cells from S5K-CTL after stimulation with E75 (*A*) or S5K (*B*) in the absence (○) or presence (●) of CH11 mAb. Equal numbers of S5K-CTL were stimulated with DCs pulsed with 0, 25, and 50 μg/ml of each peptide. The number of $CD8^+$ cells was determined by flow-cytometry using anti-CD8 mAb-FITC conjugated. *C*, Ag-induced resistance to CD95-mediated apoptosis. S5K-CTL were stimulated with autologous DCs pulsed with E75 or S5K at 5 and 25 μg/ml or control no peptide (0). CH11 mAb was added 1 h later. The number of apoptotic cells was determined 1 and 4 days later. *D*, Restimulation with E75 and S5K-induced resistance to CD95-mediated apoptosis in S5K-CTL stimulated 1 wk before with S5K. Apoptotic cells are shown in the panel subG1. Results are from one experiment representative of three independently performed experiments. Bars indicate unstimulated (■), E75 stimulated (▨), E75 + anti-Fas stimulated (■), S5K-stimulated (▨), and $S5K^+$ anti-Fas stimulated (□).

(α-Fas) (α-Fas)

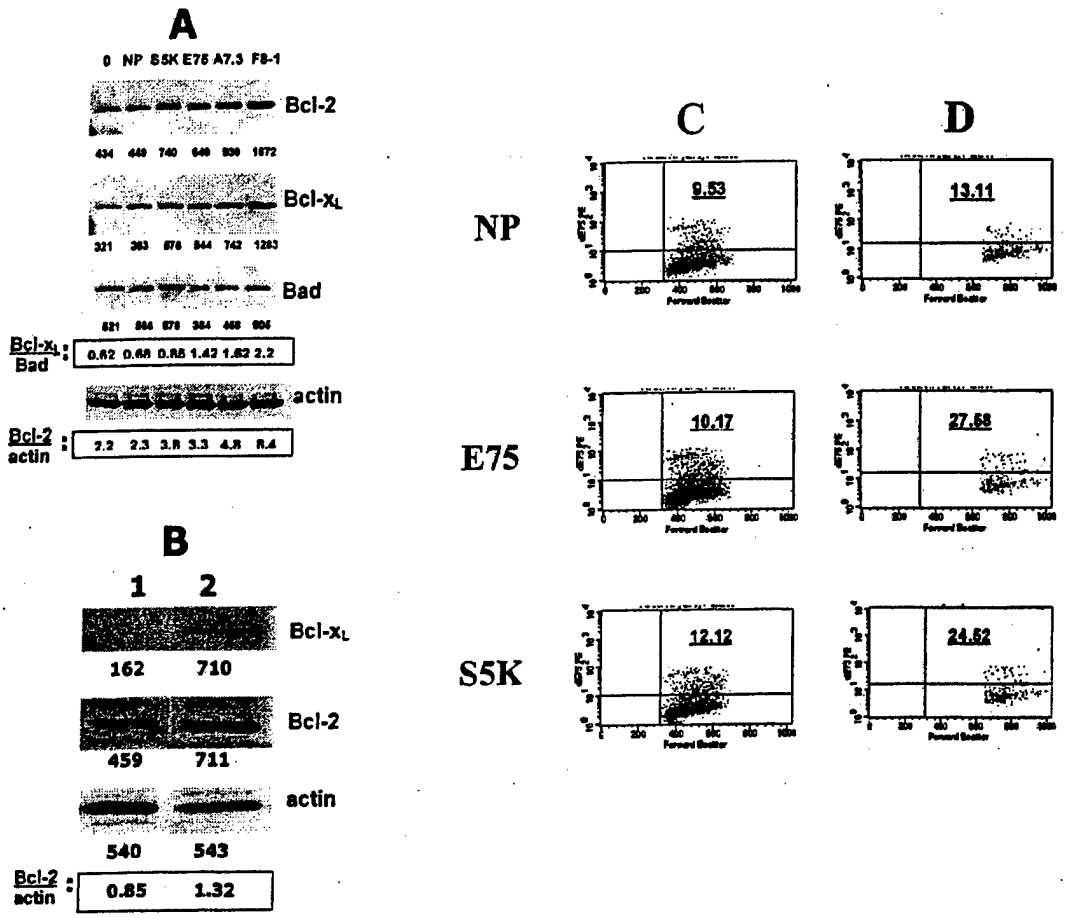


FIGURE 7. *A*, Expression levels of Bcl-family members by S5K-CTL stimulated with the indicated peptides; or *B*, with PHA for 96 h. The same blot was used for probing with all Abs. 1 indicates unstimulated; 2 indicates PHA-stimulated cells. The numbers below the bands indicate the densitometric values (pixel total × 10^{-3}) *C* and *D*, Expansion of E75$^+$TCR cells in S5K-CTL stimulated in parallel with T2-E75 (E75), T2-S5K (S5K), or with T2-NP (NP) as control for 1 wk. The presence of E75$^+$TCR cells was determined using dE75 (*y*-axis). Forward scatter (FW) is shown on *x*-axis. *C*, E75$^+$TCR cells expression in large lymphocytes (FW: 640-1000); *D*, E75$^+$TCR expression on small lymphocytes (FW: 380–600). The percentage of dNP$^+$ cells ranged from 0.1–0.5% in both populations.

after staining and after an additional 50-min incubation of dE75-
F8 stained cells in PBS (Fig. 7, *C* and *D*, and Fig. 8*A*). For further refinement, E75$^+$TCR expression and Bcl-2 expression were analyzed separately in two gated populations of smaller size (FW scatter: 380–600) and of larger size (FW scatter: 640-1000). In the small lymphocytes (Fig. 7*C*), the percentages of E75$^+$TCR cells were similar in all three stimulation groups and the E75 and S5K-stimulated S5K-CTL appeared to have similar affinities for dE75, which were stable >50 min. In contrast, in the larger lymphocytes, the percentage of E75$^+$TCR cells was higher in the E75-stimulated than in S5K-stimulated S5K cells (Fig. 7*D*). The affinity for E75 also seemed to be higher in the E75-stimulated group than in the S5K-stimulated group (Fig. 8*A*). Because E75-stimulated cells proliferated better than S5K-stimulated cells, we calculated the number of E75$^+$-TCR cells in each stimulated culture. The number of E75$^+$-TCR cells in both small and large lymphocytes stimulated by E75 was higher than in the S5K-stimulated S5K-CTL (Fig. 8*B*). The percentage increase was similar to the increase observed in CD8$^+$ cells (Fig. 6, *A* and *B*). This finding confirmed that S5K-induced CTL expanded better when restimulated with E75 than when restimulated with S5K. The levels of E75$^+$TCR and Bcl-2 in the E75-stimulated S5K-CTL in the large lymphocytes were also higher than in the S5K-stimulated S5K-CTL (Fig. 8, *C* and *D*). This suggested that stimulation of S5K-CTL with E75 resulted in changes in receptor distribution or conformation that increased the binding of dE75 as suggested by Braciale and Spencer (32). These effects were not observed in the small E75$^+$TCR lymphocytes. Bcl-2 levels were higher in the small lymphocytes after stimulation with S5K compared with E75. E75-stimulated S5K-CTL recognized E75 both as peptide and when endogenously presented by tumor (data not shown). Together these results indicate that priming CD8$^+$ cells with agonists for induction of cytolysis that are weaker than the nominal wild-type Ag followed by restimulation with the wild-type Ag can bypass induction of apoptosis either by the wild-type Ag (at priming) or by the weak agonist (at restimulation). This effect leads to increased survival and expansion of antitumor effectors.

Discussion

In this paper, we investigated the possibility of using molecular models of peptide:HLA-A2 complexes to select side chains that can induce CTL responses in T cells recognizing the HER-2 CTL epitope E75. The E75-HLA-A2 model identified several residues, Lys^1, Ser^5, Phe^8, that point upwards and may contact the TCR. We found that Ser^5 variants affected activation of T cell effector functions and produced differential levels of effector activity in CTL against the wild-type peptide E75. Modifications that removed the OH group led to variants that induced higher levels of IFN-γ at

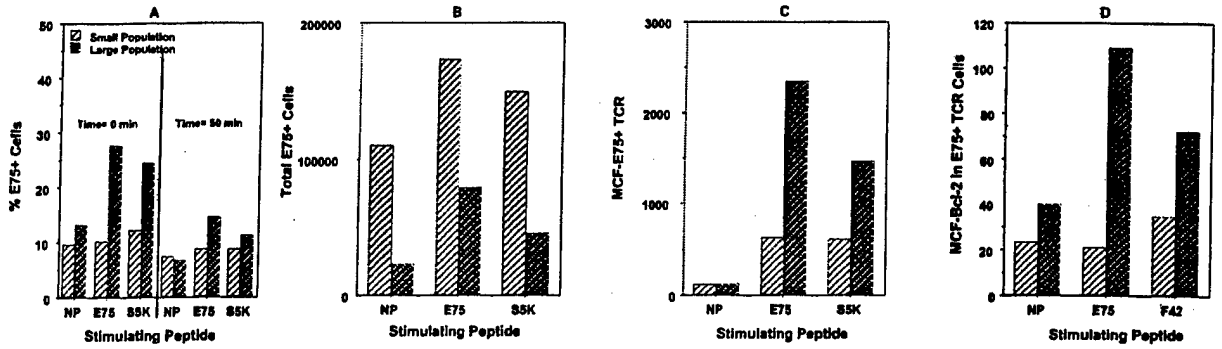


FIGURE 8. Stimulation of S5K-CTL with E75 significantly increased the number of E75$^+$TCR cells. *A*, Percentage of E75$^+$TCR cells in the large (▨) and small (▩) lymphocytes was determined immediately after staining and 50 min after washing and incubation of cells in PBS to dissociate low-affinity ($t_{1/2}$ < 50 min) TCR-dE75 complexes. Most small lymphocytes recognized E75 with $t_{1/2}$ of >50 min, while ~50% of large lymphocytes had a $t_{1/2}$ of 50 min for E75. *B*, Increase in the numbers of E75$^+$TCR cells of S5K-CTL after stimulation with E75 and S5K large (▨) and small (▩) lymphocytes. The numbers of live cells recovered after stimulation with T2-NP, T2-E75, and T2-S5K, and expansion in IL-2 were 2.7, 3.2, and 2.9 × 10^6 cells, respectively. *C*, Increased levels of expression of E75$^+$TCR in large lymphocytes stimulated with E75 compared with S5K. The differences in MCF in small lymphocytes were minimal: 202 for E75, 180 for S5K. *D*, Increased levels of expression of Bcl-2 in E75$^+$TCR large lymphocytes but not in small lymphocytes at stimulation with E75 or S5K. All determinations were performed in the same experiment. Results are from one determination representative of two with similar results.

priming. In addition, CTL primed by the variant S5A recognized E75 better in lytic assays than did CTL induced by E75. In contrast, modification of E75 by extending its side chain with an aminopropyl group lead to the S5K variant, which induced IFN-γ in two strong responders to E75 but was not a better inducer of E75-CTL-specific activity. In a third donor, weak responder to E75, the potency of E75 and S5K to induce IFN-γ was similar at priming and restimulation. S5K-CTL recognized E75 with lower affinity than E75-CTL. Only at high concentrations of E75, its recognition by E75-CTL and S5K-CTL was similar. Sequential stimulations S5A→S5A, S5G→S5G, and E75→E75 led to death rather than to CTL expansion.

A possible explanation for these effects may be provided if the effects of water (H_2O) molecules are considered. The OH group of the Ser^5 can form H-bonds with residues in TCR. Intercalation of water molecules and formation of H-bonds with the OH group of Ser may decrease the affinity of binding to the TCR, while elimination of OH group may increase the number of hydrophobic interactions. Because the sequence of the TCR and crystal structures of TCR-E75-HLA-A2 complexes are not yet available, we could not define the role of water molecules in the stimulation. Thus, deletion of the OH and CH2-OH (hydroxymethyl) groups induced death by overstimulation. Repeated stimulations with S5K minimized S5K-CTL losses due to apoptosis compared with stimulations with S5A, S5G, and E75. E75 and S5K were similar in their ability to induce IFN-γ. The signal from S5K was weaker than the signal from E75 in that S5K induced significantly lower levels of IL-2 in the S5K-CTL than did E75 (A. Castilleja and C.G. Ivanov, unpublished observations). AQ: G

Once S5K-CTL were established and were protected from apoptosis by restimulation with S5K, signals from the wild-type E75, or variants with Ala^7 side chain extended with 3CH2 groups, or Phe^8 with side chain shortened with 1CH2 group, induced even higher Bcl-x_L:Bad ratios. In S5K-CTL, E75 also increased the levels of TCR expression and Bcl-2 expression more than S5K. Considering that S5K was recognized with lower affinity than E75 by E75-induced CTL, it is possible that S5K is a weak CTL activator similar to homeostatic inducers (33, 34). A possible explanation for the low affinity of S5K-CTL for E75 is that the stimulus is not sufficiently strong to bring TCR together in the appropriate conformation for wild-type Ag recognition. This may have the advantage of extending the life of such CTL. Further studies with distinct agonists should address this question.

One important consideration now emerging from lymphocyte activation studies is that the CTL response to an Ag first expands and then contracts to bring down the number of activated effectors (35, 36). Reduction in the number of activated CTL is initiated by Ag and manifests by induction of apoptosis at restimulation a phenomenon that is amplified by IL-2 (37). The development of agonistic variants that more strongly activate antitumor effector CTL is a necessary requirement for immunotherapy. Such CTL may be useful if they can mediate immediate effects, i.e., tumor eradication upon activation. Repeated stimulations/vaccinations with strong agonistic variants may lead to depletion of highly activated effectors (38, 39). This raises concerns regarding the use of agonistic variants that are stronger than the nominal Ag in cancer vaccination for induction of central and peripheral memory CTL, because the life span of T cells activated by agonistic variants may be limited. An additional consideration emerging from activation studies is that agonist-induced effectors should survive and maintain their lytic function at encounter with the wild-type tumor Ag. We noted that CTL induced by wild-type E75 showed poor viability after two to three rounds of stimulation. This pattern of response is in agreement with the general pattern of responses to activation by self-specific T cells to avoid induction of autoimmunity (40).

Activation of antitumor effector CTL by weak agonists followed by wild-type Ag is a novel approach to promote their expansion and functional competence that has not been described before in human tumor systems. Similarly, protection from apoptosis and expansion of these cells by the self-peptide tumor Ag is also a novel finding for tumor systems. Such effectors may be useful for controlling the growth of tumors that express high levels of tumor Ag (e.g., HER-2). In addition, low-level activation of effector functions by weak agonists that can also induce homeostatic proliferation may be useful for immunotherapy after chemotherapy or radiation treatments, both of which are known to reduce leukocyte counts. This possibility is supported by studies with experimental models showing that activated low-avidity CTL that are specific for a self Ag can induce tumor rejection (40), and that stimulation of low-affinity clones can break tolerance to T cell epitopes (41, 42). Earlier studies demonstrated that differential TCR signaling

can regulate functional activation and apoptosis in T cells (43). High-strength TCR-Ag interactions lead to activation-induced cell
AQ: H death, while low-strength TCR-Ag interactions can promote death by neglect. However, depending on the nature of TCR-Ag interactions, a range of cellular responses can be induced to avoid cell death (44). Recent manipulations of such responses have involved the use of "null ligands" to attenuate the signaling by strong agonists for high-affinity CTL activation (45), the use of stronger agonists to improve the proliferative capacity of low-avidity CTL (46), and the use of molecular modeling to direct repairs in weak/partial agonists (10).

Priming a CTL response to an immunodominant epitope simultaneously results in priming to variants of the peptide sequence that the individual has not encountered (47, 48). Our previous studies demonstrated that ovarian and breast tumor-associated lymphocyte, which recognized E75, also recognized better variants S5A and S5G (49). This suggested that CTLs that recognized these variants were present in patients. The possible contribution of such clones to the immune response against tumor is still unknown (50–52). Our results show that CTL survival and effector function can be enhanced by sequential stimulation with Ag variants followed by wild-type Ag. This strategy allows the response to be followed or shifted to clones that may be endowed with better survival capacity and can differentiate to peripheral memory cells, or clones with better effector function as needed. Because S5A and S5G are stronger agonists than S5K, while F8Y and F8K appear to be weaker agonists than S5K, it will be important to determine how exposure to these variants can maintain the focus of the CTL response to the wild-type tumor Ag, and which vaccination strategy is more effective in maintaining a response against tumors in patients with persistent disease. Ongoing studies in our laboratory aim to address the effects of sequential stimulation with homeostasis inducers, strong agonists, and weak agonists in developing an antitumor response.

Acknowledgments

We thank Dr. Martin Campbell for HLPC analysis of peptides, and Dr. Christine Wogan for editing this paper. We thank the volunteers who enthusiastically donated blood for these studies.

References

1. Abrams, S. I., and J. Schlom. 2000. Rational antigen modification as a strategy to up-regulate or down-regulate antigen recognition. *Curr. Opin. Immunol. 12:85.*
2. Bownds, S., P. Tong-On, S. A. Rosenberg, and M. Parkhurst. 2001. Induction of tumor-reactive cytotoxic T-lymphocytes using a peptide from NY-ESO-1 modified at the carboxy-terminus to enhance HLA-A2.1 binding affinity and stability in solution. *J. Immunother. 24:1.*
3. Overwijk, W. W., A. Tsung, K. R. Irvine, M. R. Parkhurst, T. J. Goletz, K. Tsung, M. W. Carroll, C. Liu, B. Moss, S. A. Rosenberg, and N. P. Restifo. 1998. gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J. Exp. Med. 188: 277.*
4. Valmori, D., F. Levy, I. Miconnet, P. Zajac, G. C. Spagnoli, D. Rimoldi, D. Lienard, V. Cerundolo, J. C. Cerottini, and P. Romero. 2000. Induction of potent antitumor CTL responses by recombinant vaccinia encoding a Melan-A peptide analogue. *J. Immunol. 164:1125.*
5. Kuhns, J. J., M. A. Batalia, Y. Shuqin, and E. J. Collins. 1999. Poor binding of a HER-2/neu epitope (GP2) to HLA-A2.1 is due to a lack of interaction with the center of the peptide. *J. Biol. Chem. 274:36422.*
6. Scrody, J. S., E. J. Collins, R. M. Tisch, J. J. Kuhns, and J. A. Frelinger. 2000. T cell activity after dendritic cell vaccination is dependent on both the type of antigen and the mode of delivery. *J. Immunol. 164:4961.*
7. Fisk, B., B. Chesak, M. S. Pollack, J. T. Wharton, and C. G. Ioannides. 1994. Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2 neu proto-oncogene in vitro. *Cell. Immunol. 157:415.*
8. Salazar, E., S. Zaremba, P. M. Arlen, K. Y. Tsang, and J. Schlom. 2000. Agonist peptide from a cytotoxic T-lymphocyte epitope of human carcinoembryonic antigen stimulates production of TC1-type cytokines and increases tyrosine phosphorylation more efficiently than cognate peptide. *Int. J. Cancer 85:829.*
9. Rivoltini, L., P. Squarcina, D. J. Loftus, C. Castelli, P. Tarsini, A. Mazzocchi, F. Rini, V. Viggiano, F. Belli, and G. Parmiani. 1999. A superagonist variant of peptide MART1/Melan A27-35 elicits anti-melanoma $CD8^+$ T cells with enhanced functional characteristics: implication for more effective immunotherapy. *Cancer Res. 59:301.*
10. Degano, M., K. C. Garcia, V. Apostolopoulos, M. G. Rudolph, L. Teyton, and I. A. Wilson. 2000. A functional hot spot for antigen recognition in a superagonist TCR/HMC complex. *Immunity 12:251.*
11. Saito, N. G., H. C. Chang, and Y. Paterson. 1999. Recognition of an MHC class-I restricted antigenic peptide can be modulated by para-substitution of its buried tyrosine residues in a TCR-specific manner. *J. Immunol. 162:5998.*
12. Tourdot, S., A. Scardino, E. Saloustrou, D. A. Gross, S. Pascolo, P. Cordopatis, F. A. Lemonnier, and K. Kosmatopoulos. 2000. A general strategy to enhance immunogenicity of low-affinity HLA-A2.1-associated peptides: implication in the identification of cryptic tumor epitopes. *Eur. J. Immunol. 30:3411.*
13. Madden, D. R, D. N. Garboczi, and D. C. Wiley. 1993. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell 75:693.*
14. Garboczi, D. N., P. Ghosh, U. Utz, Q. R. Fan, W. E. Biddison, and D. C. Wiley. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature 384:134.*
15. Lee, T. V., B. W. Anderson, G. E. Peoples, A. Castilleja, J. L. Murray, D. M. Gershenson, and C. G. Ioannides. 2000. Identification of activated tumor-Ag-reactive $CD8^+$ cells in healthy individuals. *Oncol. Rep. 7:455.*
16. Anderson, B. W., G. E. Peoples, J. L. Murray, M. A. Gillogly, D. M. Gershenson, and C. G. Ioannides. 2000. Peptide priming of cytolytic activity to HER-2 epitope (369-377) in healthy individuals. *Clin. Cancer Res. 6:4192.*
17. Fisk, B., T. L. Blevins, J. T. Wharton, and C. G. Ioannides. 1995. Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. *J. Exp. Med. 181:2109.*
18. Saper, M. A., P. J. Bjorkman, and D. C. Wiley. 1999. Refined structure of the human histocompatibility antigen HLA-A2 at 26 A resolution. *J. Mol. Biol. 219: 377.*
19. Berman, H. M., J. Westbrook, Z. Feng, G. Gillian, T. N. Bhat, H. Weissig, I. N. Shindyalov, and P. E. Bourne. 2000. The protein data bank. *Nucleic Acids Res. 28:235.*
20. Peitsch, M. C., M. R. Wilkins, L. Tonella, J. C. Sanchez, R. D. Appel, and D. F. Hochstrasser. 1997. Large-scale protein modeling and integration with the SWISS-PROT and SWISS-21 the example of *Escherichia coli*. *Electrophoresis 18:498.*
21. Hausman, S., W. E. Biddison, K. J. Smith, D. Yuan-Hua, D. N. Garboczi, U. Ursula, D. C. Wiley, and W. Kai. 1999. Peptide recognition by two HLA-A2/tax_{11-19}-specific T cell clones in relationship to their MHC/peptide/TCR crystal structures. *J. Immunol. 162:5389.*
22. Knutson, K. L., K. Schiffman, and M. L. Disis. 2001. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. *J. Clin. Invest. 107:477.*
23. DiSomma, M. M., F. Somma, M. S. G. Saveria, R. Mangiacasle, E. Cundari, and E. Piccolella. 1999. TCR engagement regulates differential responsiveness of human memory T cells to FAS (CD95)-mediated apoptosis. *J. Immunol. 162: 3851.*
24. Ward, N. E., J. R. Stewart, C. G. Ioannides, and C. A. O'Brian. 2000. Oxidant-induced S-glutathiolation inactivates protein knase C-α (PKC-)—a potential mechanism of PKC isozyme regulation. *Biochemistry 39:10319.*
25. Ding, Y. H., B. M. Baker, D. N. Garboczi, W. E. Biddison, and D. C. Wiley. 1999. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity 11:45.*
26. Baker, B. M., S. J. Gagnon, W. E. Biddison, and D. C. Wiley. 2000. Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: implications for TCR signaling. *Immunity 13:475.*
27. Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of invidividual peptide side-chains. *J. Immunol. 153:163.*
28. Rammensee, H.-G., J. Bachmann, N. N. Emmerich, O. B. Bachor, and S. Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics 50:213.*
29. Kirchhoff, S., W. W. Muller, W. Krueger, I. Schmitz, and P. H. Krammer. 2000. TCR-mediated up-regulation of c-$FLIP_{SHORT}$ correlates with resistance toward CD95-mediated apoptosis by blocking death-inducing signaling complex activity. *J. Immunol. 165:6293.*
30. Mueller, D. L., S. Seiffert, W. Fang, and T. W. Behren. 1996. Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned $CD4^+$ helper T cells: a model for long-term survival of memory cells. *J. Immunol. 156:1764.*
31. Broome, H. E., C. M. Dargan, S. Krajewski, and J. C. Reed. 1995. Expression of Bcl-2, Bcl-x, and Bax after T cell activation and IL-2 withdrawal. *J. Immunol. 155:2311.*
32. Spencer, J. V., and T. J. Braciale. 2000. Incomplete $CD8^+$ T lymphocyte 2000: differentiation as a mechanism for subdominant cytotoxic T lymphocyte responses to a viral antigen. *J. Exp. Med. 191:1687.*
33. Goldrath, A., and M. J. Bevan. 1999. Low affinity ligands for the TCR drive proliferation of mature $CD8^+$ T cells in lymphopenic hosts. *Immunity 11:183.*
34. Goldrath, A. W., L. Y. Bogatzki, and M. J. Bevan. 2000. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J. Exp. Med. 192:557.*
35. Whitmire, J. K., and R. Ahmed. 2000. Costimulation in antiviral immunity: differential requirements for $CD4^+$ and $CD8^+$ T cell responses. *Curr. Opin. Immunol. 12:448.*

36. Zheng, L., C. L. Trageser, D. M. Willerford, and M. J. Lenardo. 1998. T cells growth cytokines cause the superinduction of molecules mediating antigen-induced T lymphocyte death. *J. Immunol. 160:763.*
37. Anderton, S. M., C. G. Radu, P. A. Lowrey, E. S. Ward, and D. C. Wraith. 2001. Negative selection during the peripheral immune response to antigen. *J. Exp. Med. 193:1.*
38. Combadiere, B., E. Reis, C. Sousa, C. Trageser, L.-X. Zheng, C. R. Kim, and M. J. Lenardo. 1998. Differential TCR signaling regulates apoptosis and immunopathology during antigen responses in vivo. *Immunity 9:305.*
39. Nugent, C. T., D. J. Morgan, J. A. Biggs, A. Ko, I. M. Pilip, E. G. Pamer, and L. A. Sherman. 2000. Characterization of $CD8^+$ T lymphocytes that persist after peripheral tolerance to a self antigen expressed in the pancreas. *J. Immunol. 164:191.*
40. Morgan, D. J., H. T. Kreuwel, S. Fleck, H. I. Levitsky, D. M. Pardoll, and L. A. Sherman. 1998. Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J. Immunol. 160:643.*
41. Zugel, U., R. Wang, G. Shih, A. Sette, J. Alexander, and H. M. Grey. 1998. Termination of peripheral tolerance to a T cell epitope by heteroclitic antigen analogues. *J. Immunol. 161:1705.*
42. Wang, R., Y. Wanz-Zhu, C. R. Gubaglia, K. Kimachi, and H. M. Grey. 1999. The stimulation of low-affinity, nontolerized clones by heteroclitic antigen analogues causes the breaking of tolerance established to an immunodominant T cell epitope. *J. Exp. Med. 190:983.*
43. Germain, R. M., and I. Stefanova. 1999. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu. Rev. Immunol. 17:467.*
44. Sandberg, J. K., L. Franksson, J. Sundback, J. Michaelsson, M. Petersson, A. Achour, R. P. Wallin, N. E. Sherman, T. Bergman, H. Jornvall, et al. 2000. T cell tolerance based on avidity thresholds rather than complete deletion allows maintenance of maximal repertoire diversity. *J. Immunol. 165:25.*
45. Micheletie, F., S. Vertuani, M. Marastoni, L. Tosi, S. Volinia, S. Traniello, and R. Gavioli. 2000. Supra-agonist peptides enhance the reactivation of memory CTL responses. *J. Immunol. 165:4264.*
46. de Visser, K. E., T. A. Cordaro, H. W. H. G. Kessels, F. H. Tirion, T. N. M. Schumacher, and A. M. Kruisbeek. 2001. Low-avidity self-specific T cells display a pronounced expansion defect that can be overcome by altered peptide ligands. *J. Immunol. 167:3818.*
47. Charini, W. A., M. J. Kuroda, J. E. Schmits, K. R. Beaudry, W. Lin, M. A. Lifton, G. R. Krivulka, A. Necker, and N. L. Letwin. 2001. Clonally diverse CTL response to a dominant viral epitope recognizes potential epitope variants. *J. Immunol. 167:4996.*
48. Haanen, J. B., M. C. Wolkers, A. M. Kruisbeek, and T. N. Schumacher. 1999. Selective expansion of cross-reactive $CD8^+$ memory cells by viral variants. *J. Exp. Med. 190:1319.*
49. Fisk, B., C. Savary, J. M. Hudson, C. A. O'Brian, J. L. Murray, J. T. Wharton, and C. G. Ioannides. 1995. Changes in an HER-2 peptide up-regulating HLA-A2 expression affect both conformational epitopes and CTL recognition: implications for optimization of Ag presentation and tumor-specific CTL induction. *J. Immunother. 18:197.*
50. Dudley, M. E., M. I. Nishimura, A. K. Holt, and S. A. Rosenberg. 1999. Antitumor immunization with a minimal peptide epitope (G9-209-2M) leads to a functionally heterogeneous CTL response. *J. Immunother. 22:288.*
51. Clay, T. M., M. C. Custer, M. D. McKee, M. Parkhurst, P. F. Robbins, K. Kerstann, J. Wunderlich, S. A. Rosenberg, and M. I. Nishimura. 1999. Changes in the fine specificity of gp100(209-217)-reactive T cells in patients following vaccination with a peptide modified at an HLA-A21 anchor residue. *J. Immunol. 162:1749.*
52. Dudley, M. E., L. T. Ngo, J. Westwood, J. R. Wunderlich, and S. A. Rosenberg. 2000. T-cell clones from melanoma patients immunized against an anchor-modified gp100 peptide display discordant effector phenotypes. *Cancer J. 6:69.*

Toxicity, Immunogenicity, and Induction of E75-Specific Tumor-Lytic CTL by HER2 Peptide E75 (369-377) Combined with GM-CSF in HLA-A2+ Patients with Metastatic Breast and Ovarian Cancer

James L. Murray[2], Michael Gillogly, Donna Przepiorka, Hannah Brewer, Nuhad Ibrahim, Daniel Booser, Gabriel N. Hortobagyi, Andrzej P. Kudelka, Nancy Hosken, Kenneth H. Grabstein, Martin A. Cheever and Constantin G. Ioannides

Departments of Bioimmunotherapy (J.L.M., M.E.G.), Immunology and Gynecologic Oncology, (C.G.I.), Breast Medical Oncology (N.I., D.B., G.N.H.) and Gynecologic Medical Therapeutics, (A.P.K.) M.D. Anderson Cancer Center; Center for Gene Therapy, Baylor College of Medicine, Houston, Texas (D.P.); Corixa Corporation, Seattle, Washington (N.H., K.H.G., M.E.C.)

[1]Supported in part by grants from the Department of Defense Breast Cancer Program, (DAMD 4313 and I7098, CGI), The Nellie B. Connally Breast Research Fund, and funds from Corixa Corporation, (JLM.). Peptide synthesis was supported in part by the M.D. Anderson Cancer Center Core Grant CA 16672.

[2]To whom requests for reprints should be addressed at Box 422, M D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. 713-792-4561, 713- 792-8189 (fax)

Abbreviations used in this paper: HER-2, HER-2/neu protein; DTH, delayed type hypersensitivity, PBMC, peripheral blood mononuclear cells, S.I, stimulation index, CTL cytotoxic T lymphocytes; IL-12, interleukin-12, DC, dendritic cells, TIL/TAL, tumor infiltrating/associated lymphocyte, SD, standard deviation; 3H-T dR, tritiated thymidine; α, anti. TT, tetanus toxoid; LU, lytic units; NP, not pulsed/no peptide; ST, stimulation; IVS, *in vitro* stimulation; NP-ST, stimulated with no peptide; E75-ST, stimulated with E75; T2-E75, T2 cells pulsed with E75; T2-NP, T2 cells not pulsed with peptide; DC-E75, dendritic cells pulsed with E75; DC-NP, dendritic cells not pulsed with peptide, TTP, time to progression.

ABSTRACT

To determine the toxicity and immunogenicity of the HER2/neu (HER-2), HLA-A2 restricted peptide, E75, in patients with metastatic breast and ovarian cancer, 14 patients were vaccinated with escalating amounts of E75 (100, 500 and 1000 μg) mixed with 250 μg GM-CSF as adjuvant. Each vaccine dose was administered in a total volume of 1.5 ml divided into 4 intradermal injections and administered weekly for 4 weeks, followed by monthly boosts for a total of 10 injections. Vaccinations were well tolerated without significant toxicity. Blood was drawn prior to, at 8 weeks, and up to 13-16 months post-vaccination for measurement of cellular immunity. Seven of eight patients tested had significant DTH to E75 defined as > 5mm induration. PBMC from 5 of 9 patients tested proliferated to E75 with S.I. of $\geq$ 2.0. Of eight vaccinated patients tested for induction of a CTL response, four responded to stimulation by autologous DC plus cytokines by eliciting E75-specific lytic activity consistent with the presence of activated/memory cells, two others after IVS with E75 + IL-12 $\pm$ αCTLA-4, while two others did not respond. Four patients with E75 specific CTL present specifically recognized E75 on indicator tumors as demonstrated by cold-target inhibition of tumor lysis. These four patients showed E75-specific IFN-γ production. PBMC from three of these patients proliferated to E75, but S.I were higher in the pre-vaccine samples. All four patients showed DTH responses to E75. These results demonstrate that vaccination with E75+GM-CSF can induce both peptide-specific IFN-γ and epitope specific CTL which lyse HER-2^{+} tumors in Stage IV patients.

INTRODUCTION

HER-2/neu (HER-2) proto-oncogene is amplified on tumors in from 20-30% of patients with breast and ovarian cancer. Since HER-2 is an overexpressed nonmutated "self" protein it was believed that it will not be immunogenic in humans. However, HER-2 overexpression was postulated to lead to a higher level of T cell epitope precursors and of epitopes, which can activate T cells in vivo, and *in vitro* (**1**). In confirmation of these hypotheses CTL specific for HER-2 epitopes in the 968-984 area have been identified in ovarian cancer tumor associated lymphocytes (**2**). Antibodies reactive with HER-2 have been detected in the serum of breast cancer patients (**3**). These and additional studies demonstrated pre-existing T-cell immunity in patients with HER- 2 positive cancers (**4, 5**) raising the possibility of using HER-2 as a target for cellular immune responses to tumors.

Cancer vaccines that target self tumor antigens are often weaker immunogens for $CD8^+$ CTL induction than foreign antigens (**6**). In general peptides are considered weak immunogens for CTL recognizing endogenous epitopes (**7, 8**). This holds true for peptide vaccines in melanoma, ovarian and breast carcinoma (**9, 10**). In a few instances examples of peptide-induced tumor cytolytic T cells were reported in vaccinated ovarian and breast cancer patients (**11-13**). Peptide vaccines are less likely to induce low dose tolerance and likely can overcome Ag ignorance due to the presence of the antigenic epitopes at 50-100 fold higher concentrations than whole proteins. (**14**) A major concern with tumor-Ag-specific CTL induction by peptide vaccines is that the resulting CTL in many instances do not recognize tumor cells, because of their low affinity for endogenous Ag, (**9**) although the TCR repertoire is present, both in patients and healthy donors (**15-17**). An alternative possibility is that local immunization with μM amounts of CTL epitopes may induce apoptosis by overstimulation of existent high-affinity Ag-specific CTL (**12**). In patients with advanced metastatic disease, the function of these high-affinity CTL it is still unclear (**16**). This raises the questions whether peptide vaccines can induce immune responses in patients with advanced disease (Stage IV), whether the induced responses are limited only to activation of cytokine elaboration or can also activate Ag-specific cytolytic activity against HER-2 expressing tumor cells. Induction of IFN-γ and perforin synthesis appears to be mediated by distinct signaling pathways originating from the TCR. In addition, the questions of specificity of *ex vivo* isolated CTL, of duration of tumor lytic E75-specific CTL and of the frequency of these response in vaccinated patients have not yet been investigated.

We demonstrated that CTL expanded from tumor associated lymphocytes from ovarian and breast cancer specifically recognized $HER\text{-}2^+$ tumors (**2, 18**). Recognition was associated with several CTL epitopes on HER-2 mapped by peptides C85 (971-979), and E75 (369-377:KIFGSLAFL). Parallel studies demonstrated an additional epitope GP2 (**19**). Peptides corresponding to C85 and E75 induced CTL that lysed $HER2^+$ HLA-A2+ tumors suggesting that C85 and E75 are immunogenic in healthy individuals (**20, 21**).

E75 was the antigen of choice for this vaccine since it is a dominant CTL epitope. The optimal adjuvants to use with peptide vaccines have not yet been determined. Studies in rats showed enhancement of DTH response to HER2 peptides vaccines in which GM-CSF was added (**22**). GM-CSF is one of the most effective cytokines for activating DC (**22, 23**). For these reasons it was selected for use together with E75 in a phase I trial of breast and ovarian cancer patients.

The primary objectives of this study were to determine the vaccine toxicity and its ability to induce E75-specific tumor-lytic CTL in breast and ovarian patients with limited Stage IV disease and the duration of this CTL response. An additional objective was to define approaches which allow detection of *in vitro* effector responses to vaccine by *ex vivo* activated CTL. Towards this goal IL-12 and α-CTLA-4 were used to co-stimulate IFN-γ production and cytolytic responses to E75.

PATIENTS AND METHODS

Subjects: Patients with Stage IV breast and ovarian cancer were eligible for study (Table 1). All patients gave written consent to participate in the study as mandated by the Surveillance Committee at M. D. Anderson Cancer Center. Patients had an ECOG performance status of 0-1 and were refractory to standard chemotherapy and/or local radiotherapy. They had been off all chemotherapy for a minimum of 3 weeks prior to study. Prior to vaccination all patients were tested for immunocompetence using a battery of 4 skin tests to recall antigens; mumps, tetanus toxoid, histoplasmin and Candida Albicans. To be eligible for vaccination at least 2 of the 4 tests had to be positive as defined by erythema and induration of ≥ 5mm in diameter. Patients had to have tumor, which overexpressed HER2, and peripheral blood lymphocytes, which were positive for HLA-A2 (see below). The use of immunosuppressive drugs such as corticosteroids was prohibited.

Fourteen patients, 13 with breast cancer and 1 with ovarian cancer, were eligible to participate in the trial. Mean age was 50 years. All patients had received 2 or more chemotherapy regimens and either had stable disease, complete, or partial remissions prior to receiving vaccinations. Six had received previous hormonal therapy. Patients had one or two sites of evaluable/measurable disease. Patient 10 was vaccinated after complete recovery of lymphocyte count following high dose chemotherapy plus autologous peripheral blood stem cell transplant.

Vaccination Schedule: GMP quality E75 peptide was synthesized and purified by Corixa Corporation. Vials of E75 were supplied at concentrations of 100, 500 or 1000μg/ml in 2.2ml of 10mM sodium acetate buffer (pH4.0). GM-CSF (Sargramostim) was purchased from Immunex Corporation as sterile, preservative-free lyophilized powder in vials containing 500μg. Immediately prior to injection, 1ml of E75 was mixed with 250g (0.5ml) of GM-CSF for a total volume of 1.5ml (**12, 23**). Patients received intradermal injections divided among all 4 extremities (approximately 0.4ml/injection) weekly for 4 weeks and then monthly for a total of 10 injections. Five patients received 100μg peptide, 5 received 500μg, and 4 received 1000μg. Patients had to receive a minimum of 5 injections over 2 months to be eligible for determination of immune reactivity to E75 and tumor response. Patients were observed up to 1 hour and 24 hours post injection for significant side effects. Acute and chronic toxicity were graded according to NCI common toxicity criteria. Although not an aim of this study, patients had repeat X-rays, CT scans and physical exams at every two month intervals to evaluate any anti-tumor activity or disease progression using traditional criteria (12).

Screening studies required for patient eligibility: (a) HER2 expression: HER2 expression was examined on paraffin block tumor specimens using IMPATH® kits with methods as described by the manufacturer. Eligible patients had 2+ or greater staining based on a graded

intensity of 1-4, and at least 20% of tumor cells staining positively for HER2/neu. **(b) HLA-A2 positivity:** HLA-A2 expression was determined with patient PBMC isolated from heparinized blood using Ficoll gradients. Immunoflourescence experiments to examine for the presence of HLA-A2 on PBMC were performed as described using mAb BB7.2 (anti HLA-A2) from supernatants of hybridomas obtained from the ATCC (Rockville, MD). The number of fluorescent cells and the fluorescence intensity were examined using an EPICS-V Profile Analyzer (Coulter Corporation, Hialeah, FL). **(18)**

Immunologic Monitoring: (1) Lymphocyte proliferation to E75: PBMC collected from peripheral blood drawn prior to vaccine treatment and at 8 weeks, just prior to the 5th vaccination, were cultured in quadruplicate in 96-well flat-bottom microtiter plates (Costar Corp, Cambridge MA) at a concentration of $2.5x10^5$ cells/well in 100µl of complete RPMI-1640 medium with L-glutamine (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Sigma, St. Louis, MO). E75 was added at a final concentration of 25µg/ml. A weak HLA-A2 binding HER2 peptide, E72 (HER-2: 828-836, QIAKGMSYL), was used at the same concentration as specificity control. One µg/ml of PHA, 5µg/ml TT (positive controls) or no peptide were positive and negative controls, respectively. Cells were cultured for 6 days at 37°C in 5% CO^2. [^{3}H] Tdr at lµCi/well was added 16-18 hours before the end of culture as described **(12)**. Results were expressed as cpm [^{3}H] Tdr incorporation and as a stimulation index (SI) calculated as: (average cpm of lymphocytes cultured with peptide or positive controls) ÷ cpm of (lymphocytes cultured in without peptides).

(2) Delayed type hypersensitivity (DTH) responses to E75: In 7 patients, 50ug of E75 peptide was injected subcutaneously along with a normal saline control at 8 weeks immediately prior to the 5th vaccination to determine DTH response to peptide. Responses were not tested prior to vaccination because of the concern that the Ag might serve to "prime" lymphocytes to E75. Responses were recorded as the maximum diameter of skin induration in mm. Values > 5mm induration (i.e. that used to grade recall antigens) were considered positive.

Interferon gamma secretion as measured by ELISPOT: Enumeration of IFN- producing cells was done by counting under magnification of 20 with a stereomicroscope (Leitz GZ6; Leitz, Wetzlar, Germany). Only large spots with fuzzy borders were scored as positive. Responses were considered significant if a minimum of five cells were present per well, additionally, this number was at least twice that in negative control wells.

PBMC obtained from patients pre and post vaccination were cultured at a concentration of $1x10^6$ cells well _____ 48 well plate in 1ml.well RPMI + 10% human AB serum. Cells were stimulated with 1µg/ml Flu M1 peptide or E75 (10µg/ml) with or without IL> (10mg/ml). IL-2 (1mg/ml) was added on days 1 & 5 of culture. Cultures were tested by ELISPOT on day 7 against autologous PBMC plus (m1 5µg/ml). E75 (10µg/ml) HIV RT peptide (5 µg/ml) or PHA (3 µg/ml) as negative and positive controls, respectively.

(3) IFNγ secretion in supernatants: Supernatants collected at 24 and 48 h from lymphocytes following repetitive <u>in vitro</u> stimulations with peptide (see below) were used for measurement of IFNγ levels in duplicate using an ELISA kit with a sensitivity of 10pg/ml (Biosource, Camarillo, CA). Differences were considered significant if higher than 50pg/ml.

(4) Detection of CTL induction by vaccine; Generation of PBMC-derived DC: Patient PBMC were added to 24 well flat bottomed plastic plates (Costar). After 2 hrs. incubation at 37°C in RPMI-1640 (serum free), non-adherent cells were removed by repeated washings and frozen in liquid nitrogen at a concentration of 10 x 10^6 cells/ml in FCS with 5% DMSO. Monocyte-derived DC were obtained by culturing the adherent cell population in complete RPMI supplemented with 1000 IU/ml GM-CSF and 500 IU/ml IL-4 (Bioscience International) (DC medium) for 5 days (**19**). DC were detached with 0.5mM PBS-EDTA and tested for purity by staining using PE-conjugated anti CD13 mAb (Caltag Laboratories, San Francisco, CA) followed by FACS analysis. Over 90% of cells were CD13 positive. DCs were then replated at $1.2x10^5$ cells/well in 24-well plates and pulsed with 25 μg/ml E75 in serum- free medium for 4 h. TNF-α (Chiron Corporation, Emoryville, CA) was added at a concentration of 50 U/ml to DC for the last hour to stimulate DC maturation and antigen presentation.

Responder PBMC were resuspended in RPMI 1640 containing 10% human serum (HS) and added to autologous DC at 1.5 x 10^6 cells/ml (effector: stimulator ratio of 12.5:1) (**21**). In some experiments IL-12, a gift from Dr. Stanley Wolf, (Genetics Institute, Cambridge, MA), was added 60 minutes later at a concentration of 3IU/ml (300pg/ml) aiming to enhance primary antigen stimulation. Following expansion in IL-2, lymphocytes were washed twice and incubated without IL-2 for 20h in complete medium. They were then tested for their ability to lyse ^{51}Cr- labeled T2 cells, pulsed with various concentrations of E75 (5-25μl/ml), using as specificity control a high HLA-A2 affinity HER-2 peptide: E71(HER-2, 799-807: QLMPYGCLL) (**18**). HLA-A2 expressing SKOV3 ovarian tumor cells, developed in this laboratory, SKBR3A2 breast tumor cells (a gift from Dr. M. Disis, Seattle, WA) and freshly isolated ovarian tumor cells (HLA-$A2^+$, HER-2^+) were used in cold-target inhibition experiments. Results are expressed as % specific lysis as described. (**2, 18**) In some experiments lymphocytes were stimulated three times with DC-E75 in the presence of IL-12 for up to 3 weeks prior to testing for CTL activity. Responses were considered positive when the mean ± S.D. % specific lysis of T2-E75 was significantly greater than the mean ± S.D.% specific lysis of T2-NP or T2-E71 as described (**12**).

Data analysis: Statistical analysis was performed using unpaired Students' t-test as well as analysis of variance (ANOVA) for three or more groups.

RESULTS

Toxicity: There was no grade 3 toxicity to E75 and GM-CSF vaccine. (**Table 2**). The majority of patients had mild pain and erythema at the injection site. Low-grade fever was the second most frequent side effect, followed by grade 1 nausea, fatigue, myalgias, itching at the injection site, and back and abdominal pain. There was one episode of grade 2 chills, 7 episodes of headache, and 2 episodes of moderate ulceration at the injection sites, all occurring in patient No. 6. No cumulative toxicities were observed. Side effects to vaccine were not dose dependent.

Patient status: There were no tumor responses observed. The number of vaccinations received and the time to progression (TTP) are shown in Table 1. All except patient 10 progressed prior to receiving all 10 injections, with a mean TTP of 11 months. Patient 10 remained disease free for 52 weeks until relapsing in bone marrow. At the patient's insistence, chemotherapy and

Herceptin were reinstituted after completing all vaccinations; hence the duration of remission due to vaccine alone could not be assessed.

DTH responses to E75 peptide: Seven of 8 patients tested (5, 6, 7, 8, 10, 11, and 14) had positive DTH responses to E75. Patient 4 had no response (Table 1). There were no responses to the saline control. The pattern of responses to TT was similar with the pattern of responses to E75.

Lymphocyte proliferation to E75: Table 3 shows the cpm [^{3}H] Tdr incorporation and S.I. for lymphocytes from 11 of 14 patients who had PBMC tested *in vitro* to E75. Data shown are derived from patients' PBMC stimulated prior to vaccination and at 8 weeks after vaccination. Patients 1 and 3 progressed prior to 8 weeks and did not have post vaccine blood samples drawn. Patients 4,13 and 14 did not have a high enough lymphocyte yield from blood drawn for pre testing due to difficulties in venous access and did not return for post testing due to significant disease progression with poor performance status prior to 8 weeks. Five of nine patients' PBMC proliferated to E75; cpm were significantly increased over proliferation in medium alone. Post vaccination patient 6 PBMC proliferated upon *in vitro* exposure to low affinity peptide E72. There were no significant differences in proliferation to E75 from PBMC studied pre versus post vaccination in 3 patients, in two patients there was an increase in S.I. after vaccination, while in 4 patients there was a decrease in S.I. in post vaccination PBMC, even though significant response to E75 was seen prior to vaccination. S.I.s ranged from 0.8 to 10.9 (mean + S.D.= 2.9 ± 2.3) . S.I.s to PHA ranged from 2 to 106. Due to wide variations between patients S.I.s pre treatment values did not differ significantly from post treatment (pre = 45 ± 43; post = 26 ± 26; $p > 0.05$). Similar results were observed for TT (pre = 6 ± 7; post = 4 ± 3; $p > 0.05$).

In essence 6 of 7 patients who had positive proliferation responses either pre or post vaccination had positive DTH. Of the seven patients with positive DTH responses to E75, four (7,8,10, and 11; Tables 3 and 5) had positive proliferation responses to peptide prior to vaccination and four had proliferation to E75 post vaccination (6, 8, 10, and 11). Three of five patients (7, 8, 10) had no significant change in proliferation post-vaccination compared to pre-vaccination; two of the five (11, 12) had significant ($p=0.04$) decrease in proliferation post treatment compared to pre-treatment.

IFNγ secretion as measured by EKLISPOT: Patients 4-9 has IFNγ secretion measured in lymphocyte usine ELISPOT. There was not detectable IFNγ to E75 despite strong responses to the dominant influenza CTL epitope from matrix protein. M1, (positive control) in lymphocytes from all patients obtained pre or post vaccination. For example, a representative experiment with donor 4 post-vaccination showed 18 ± 5 spots/2.5 x 105 PBMC and $43\pm/5$ x 105 PBMC for M1 stimulated cells, but less than 5 sports for E75, suggesting that the frequency of IFN-γ secreting cells in the absence of IL-12 was less than one in 105 cells. Thus E75 along added to PBMC was a weak inducer of IFNγ in vitro.

There was no IFNγ secreted in response to HIV-RT, whereas responses to PHA were > 200 spots/well (data not shown) which is above the upper range of measurement for the assay.

Measurement of IFNγ secretion in supernatants ± IL-12: To determine whether the Th1 cytokine IFNγ was produced from peptide pulsed lymphocytes blood was drawn prior to disease progression on patients 6, 7, 9, 10, 11, and 14. Purified PBMC were stored frozen in liquid nitrogen. Patients 10 and 11 also had additional PBMC collected and frozen prior to vaccination. PBMC were thawed and separated into DCs and non-adherent lymphocytes as described, and incubated with E75 or medium (see methods). After one or two rounds of *in vitro* stimulation (IVS) supernatants were collected at 24 and 48 hours and frozen for later analysis of IFNγ secretion. In the absence of IL-12, only two of six (10,14) patients responded by IFN-γ secretion. In the presence of IL-12, five of six patients responded to E75 *in vitro* by IFN-γ secretion. In one patient, (No. 11) vaccination induced a significant increase in response to E75. Of three patients who had no measurable IFN-γ after one IVS with E75-pulsed DC (6,7,9), IL-12 induced measurable IFN-γ levels in 2 of the 3 patients (65 and 106.3 pg/ml, respectively) **Table 4**. Although comparison of ELISPOT to ELISA may not be relevant, therefore, these patients (6,7,9) also demonstrated no IFNγ secretion to E75 using ELISPT. Patients 10, 11 and 14 also had significant increases in IFNγ secretion following 2 IVS with E75 in the presence of IL-12: mean ± SEM : 605.4±83.6 pg/ml compared to lymphocytes incubated without IL-12 (75.9±38.1 pg/m1; $p < 0.003$). This value was also significantly higher than for lymphocytes incubated without peptide (157.7±68.7pg/m1; $p<0.006$). Collectively these results suggest that E75-responsive T cells were present in these patients. It is possible, although needs to be investigated that in Stage IV patients E75 was a weak immunogen which could not activate APC and required costimulation by IL-12 to induce detectable levels of IFNγ.

E75 specific cytolytic activity: The objectives of these studies were four fold: *(a)* to determine whether patients with metastatic breast cancer developed specific cytolytic responses against E75 and of tumor cells expressing this epitope: *(b)* whether E75-specific tumor-lytic memory CTL were present in some of these patients and could be detected *in vitro*; *(c)* whether vaccination with E75 enhanced the specific-lytic activity of these CTL; *(d)* whether the use of IL-12 at priming enhanced the lytic activity against tumor of CTL from E75+GM-CSF vaccinated patients. To determine CTL activity against T2 targets pulsed with peptides E75 (T2-E75), negative control HER2 peptide E71 (T2-E71), or experimental negative control, T2 not pulsed with peptide (T2-NP) as well as HLA-A2 transfected tumor cell lines SKOV3 (ovarian) and SKBR3 (breast), lymphocytes were harvested following 1 round of *in vitro* stimulation IVS (patients 6, 7, and 9) or 3 IVS (patients 10, 11, 12, 13, and 14) and subsequently tested for lytic activity in 4 and 20h ^{51}Cr release assays.

CTL responses by Patient No. 7. Patient No. 7 was tested for CTL responses to E75 four weeks after the 7th vaccination. To increase the sensitivity of detection of E75-specific CTL autologous PBMC were primed *in vitro* with DC-E75 plus IL-12. The results in **Fig. 1A**, demonstrated that *in vitro* priming with E75+IL-12 did not induce significantly higher E75-specific lytic activity than NP+IL-12. This activity increased when α-CTLA-4 was present ($p<0.01$), suggesting that a part of E75-reactive CTL were tolerized. INF-γ secretion at 20h also increased in the presence of α-CTLA-4 from 65 to 175 pg/ml. There was no specific recognition of E75 by DC-NP stimulated PBMC suggesting that *ex vivo* activated CD8+ cells endowed with lytic function were either absent or below the levels of detection or that required E75-stimulation for activation of lytic function. Cold-target inhibition experiments showed that T2-E75 inhibited lysis by E75+IL-12+αCTLA-4 ST cells of an HLA-A2$^+$ HER-2$^+$ breast tumor significantly better than T2

E71. (38% inhibition and 12% inhibition at E:T ratios of 10:1 and20:1, respectively, **Fig. 1B**) indicating that a subpopulation of *in vitro* E75-primed CTL recognized endogenously presented E75. To obtain a general estimate of the proportion of the tumor lytic E75-specific cells we calculated lytic units (LU). LU for tumor lysis by CTL in the presence of T2-NP or T2-E71 as inhibitors were 24. $4/10^6$ cells while in the presence of T2-E75 as inhibitors they were 17. $6/10^6$ cells, indicating that 28% of the tumor lytic effectors recognized endogenous E75.

CTL responses by patients 6 and 9: Lymphocytes were collected 2 weeks following the last vaccination. IVS was performed with dendritic cells not pulsed with peptide (DC-NP) and dendritic cells pulsed with E75 (DC-E75) in the presence of IL-12. The results show that cells stimulated by DC not pulsed with peptide (NP-ST) from both patients recognized E75 better than the control HER-2 peptide E71. One IVS with DC-E75 lead to significantly decreased recognition of E75 ($p<0.05$). E75 recognition was similar (No.9) or lower (No. 6) than of E71 (**Fig. 2A, B**). This suggested that a subpopulation of *ex vivo* activated E75-reactive CTL were present in both patients. Additional *in vitro* stimulation with E75 lead to its decreased recognition raising the possibility that E75 induced apoptosis of effector CTL.

CTL responses by patients 10 and 11: Preliminary experiments with PBMC from patients 10 and 11 collected prior to treatment and after the last vaccination, respectively, showed that *in vitro* priming with DC-NP or DC-E75 was insufficient to induce detectable levels of cytolytic activity (data not shown). These results suggested that the frequency of E75-specific CTL in these patients may be lower than in patients 6, 7 and 9. For these reasons, the ability of these vaccinated patients to maintain lytic responses to E75 and HLA-$A2^+$ HER-2^+ tumors over time was determined after three consecutive *in vitro* stimulations with E75 pulsed on autologous DC.

CTL responses by patient No. 10: In experiments performed using PBMC collected five months after last vaccination we confirmed that IL-12 was required to induce detectable E75-specific lytic activity at IVS with DC-E75. αCTLA-4 antibody did not increase the CTL activity over the levels of lytic activity induced in the presence of IL-12. However, E75-specific CTL activity by DC-NP $\pm$ IL-12 stimulated PBMC was not detected (**Figure 3A**). This raised the question whether activated/memory peripheral effectors were present in insufficient numbers to be detectable in this assay. IVS of prevaccine lymphocytes from this patient did not resulted in CTL. Thus, the CTL after *in vitro* recall with E75 were similar with those of patient 7, but not with those of patients 6 and 9. To address the duration of the presence of tumor lytic CTL PBMC collected nine months after the last vaccination were stimulated with E75 presented by autologous DC. Lytic activity against tumor was tested after three *in vitro* stimulations with DC-E75. To address whether these cells recognized endogenously presented E75, we performed cold-target inhibition of lysis, using as target the ovarian tumor SKOV3.A2. The results in **Fig. 3B** show that in 5h assay at a high E:T ratio of 30:1, T2-E75, inhibited lysis of SKOV3.A2 cells by 16.6% compared with T2-NP. Again, calculation of LU indicated that 25% of the tumor lytic effectors recognized endogenous E75 ($LU/10^6$: inhibitor (T2-NP) = 83.4 vs $LU/10^6$: inhibitor (T2-E75) = 62.5).

To address whether memory effector CTL persisted in this patient, the experiments were repeated with PBMC collected 13 months after last vaccination. To enhance the type 1 response inducing ability of patient's DC in addition to IL-12, αIL-4, αIL-10, and αTGFβ mAb were added in all stimulation cultures. After three consecutive IVS with DC-NP and DC-E75 the

resulting cells were tested in CTL assays. To assure that the number of effectors did not change subsequent to changes in CD8:CD4 ratios, % expression of $CD8^+$ cells was determined in both effector populations and the effector numbers were adjusted at equal $CD8^+$ cells numbers in both assays.

The results in **Fig. 4A** show that both three IVS (3xNP-ST and 3x E75-ST) cells recognized T2 cells pulsed with exogenous E75. Recognition of E75 at the lower concentration of 5μM was higher when 3xE75-ST were used as effectors than when 3xNP-ST cells were used as effectors. Thus, E75-specific CTL were present in this patient 13 months after the last vaccination. Their reactivation required the presence of autologous MHC, and was dependent on cytokines used but did not require, although was enhanced by, restimulation with E75.

This finding suggested that E75-specific "memory-like" CTL were present in Patient No. 10. To address whether NP-ST "memory-like" CTL and E75-ST CTL differed in their lytic potential, the CTL assay was continued for up to 20h. The results in **Fig. 4B** show that the lytic activity of 3xE75-ST cells increased over a 20h period, but the lytic activity of 3xNP-ST cells declined. This raised the possibility that E75-specific "memory-like" CTL in this patient were endowed with weak lytic activity, and restimulation with E75 was required to activate their lytic function. Cold target inhibition of tumor lysis experiments showed similar results with the experiments performed four months earlier (**Fig. 4C**). In 5h assay, T2-E75 inhibited lysis by both NP-ST and E75-ST effectors compared with T2-NP. This supported the hypothesis that E75-specific cytolytic T cells recognizing tumor were present and their reactivation did not require E75. *In vitro* stimulation with E75 increased tumor lysis. 4xE75-ST effectors also lysed the breast tumor line SKBR.3.A2 (**Fig. 4.D**). T2-E75 induced 81% and 47.2% inhibition of lysis, respectively, compared with T2-NP, confirming that these cells recognized endogenously epitopes presented by the tumor.

To confirm that vaccination induced E75-specific CTL, we repeated the experiment using as responders plastic non-adherent PBMC of patient No. 11. Responders were collected before and 16 months after the last vaccination. At this time, the patient had progressive disease. In previous experiments we found that E75-specific CTL were undetectable in the PBMC collected before vaccination even when E75+IL-12 were used. Addition of αCTLA-4 at priming with DC-NP and DC-E75 did not increase E75 recognition: % specific lysis by NP-ST cells=35.4± 3.6(NP) *vs* 40.5±1.2(E75), while by E75-ST cells=37.7±1(NP) *vs* 47.9±2.5(E75). Targets are indicated in the parentheses. αCTLA-4 induced specific lysis was 10.2± 2.5. "Memory-like" CTL effectors activated by DC-NP showed a similar pattern of reactivity with CTL effectors of Patient 10 (i.e., weak specific killing in the 5h but not in the 20h CTL assay). These results suggested that "memory-like" effectors were induced in this patient by vaccination, since they were absent in the PBMC collected before vaccination tested in parallel.

The lytic activity of cells from patient No. 11 stimulated three times with DC- E75 or DC-NP is shown in **Fig. 5A-D**. **Fig. 5A** demonstrated that NP-ST PBMC collected before vaccination had no lytic activity against E75 in the 5h CTL assay and that lytic activity did not significantly increase after *in vitro* E75 stimulation. This confirmed the results of the previous experiment. PBMC collected after vaccination (**Fig. 5B**) showed little increase in E75 lytic activity in the 5h CTL assay. There was a significant increase in lytic activity for NP-ST at 5h (from 13.2 ±3.9 to

21.4±0.3; $p<0.05$) implying the presence of memory cells in this patient. This value was also markedly increased above pre values (3.4±3.6; $p<0.001$).

In contrast to what was observed after 5h incubation, PBMC collected before vaccination and incubated for 20h with targets specifically lysed T2-E75 (increase of 75%; 0 μg E75 (NP) compared to 5ug/ml E75; $p<0.01$). Similarly, lysis of T2-E75 by post-vaccine E75-ST PBMC was significantly higher than for T2-NP (57.7±5.0% versus 32.2±7.2%;$p<0.01$). Lysis was also increased for E75-ST + IL-12 (58.7±2.3%;$p<0.001$),but the lysis was not increased over that of E75-ST cells minus IL-12 (**Fig 5C, D**).

To confirm that E75-induced CTL recognized tumors, we repeated the cold-target inhibition experiments with Patient No. 14. PBMC were collected three months after last vaccination and stimulated in parallel with DC-NP, DC-E75 and DC-E75+IL-12. A caveat of this experiment was that autologous DC were not sufficient for the entire stimulation protocol, thus allogeneic HLA-A2 matched DC from healthy donor were used as APC for all the stimulation groups. CTL from all three stimulation groups expressed E75-specific lysis (not shown).

Cold target inhibition experiments (**Fig. 6A, B**) showed that T2-E75 inhibited lysis of SKOV3.A2 cells by 3x E75-ST but not lysis by 3x NP-ST cells suggesting that E75-specific memory CTL were absent from this patient. When IL-12 was present at the time of *in vitro* priming with T2-E75, resulting 3xE75+IL-12ST-CTL showed an even higher increase in inhibition of lysis compared with E75-ST-CTL. These results were similar when the assay was extended for 20h (Fig. 7B). This experiment confirmed that a subpopulation of E75-ST cells lysed tumors overexpressing HER-2. CTL which were stimulated in the presence of IL-12 showed a higher avidity for the E75, as evidenced by the higher inhibition of lysis in the E75+IL-12 group, compared with the E75 alone group.

CTL responses by patients 12 and 13: Patients, No. 12 and No. 13 showed no E75-specific CTL responses after three IVS with DC-E75 and with DC-NP, suggesting that E75-specific CTL were either absent or they could not be expanded by DC-E75 to numbers where their cytolytic activity could be detected. The second possibility is more likely since E75-specific CTL could be recalled in Patient 13 by one IVS with E75 agonist-variants presented by HLA-A2 matched DC or T2 cells in the presence of IL-12 (**24**). The ability of these variants to activate E75-specific CTL was confirmed with Patients 10 and 11 (**24**).

In summary, of the eight vaccinated patients tested for induction of a CTL response to E75 by recall *in vitro* with E75, four (No. 7, 10, 11 and 14) showed a specific lytic response to E75. E75 specific CTL responses correlated with significant increases ($p<0.01$) in IFN-γ secretion in two patients with and two without the addition of IL-12 (Table 4). In addition, three of three vaccinated patients tested contained a population of CTL whose lytic activity against tumors was inhibited by T2-E75 indicating that they recognized endogenously presented epitopes by the tumor. In four of these eight patients (No. 6, 9, 10,11) E75-specific CTL were detected after stimulation with DC-NP *in vitro* either after priming or restimulation, suggesting the presence of E75-specific "memory-like" cells in patients with metastatic breast cancer.

A summary of all immunologic tests performed for each patient is shown in Table 5. Eight patients had results from at least 2 of 4 assays. Excluding patient 10 from analysis due to additional treatment post vaccination, the mean + S.D. of TTP for 8 patients with either positive DTH responses to E75, proliferative responses to E75, or both was slightly greater (10.7 $\pm$ 4.8 weeks) than for 5 patients who had negative responses to either DTH, proliferation or both (5.7 $\pm$ 3.7 weeks; $p = 0.06$). Five of the 8 also had either specific CTL precursors for E75 and/or IFNγ production with the addition of exogenous IL-12.

DISCUSSION

The most important findings of the present vaccine study in Stage IV patients were: (1) repeated vaccinations with E75 plus GM-CSF were well tolerated. (2) in 4 of 8 patients E75+GM-CSF induced epitope specific tumor-lytic CTL; (3) such CTL were present in several patients for 1 to 12 months after completion of vaccination; (4) these CTL could be activated by self-MHC in the presence of inflammation-inducing conditions to produce IFNγ and to recognize and lyse tumor cells expressing E75. This functional behavior may be consistent with peripheral memory-effectors.

Although the patients with Stage IV disease are not considered the most promising group for treatment by cancer vaccines, compared with patients with disease remission or less advanced disease, this group conceivably could benefit the most from immunotherapy, since the applicability of other approaches is limited. The fact that Ag-specific tumor lytic CTL were detected after vaccination may address concerns that vaccination with 100 - 1000μg/ml of peptide can induce deletion of tumor-lytic effectors. Our study demonstrated that E75-specific cytolytic effectors were induced by E75, were present in the peripheral circulation, and could be detected after one recall with DC+E75, or DC alone in four of six (67%) of patients tested. Overall they were detected in a high proportion (6 of 8, 75%) of patients tested. The E75-specific repertoire was maintained over time, since such cells could be detected at recall with DC-NP or DC-E75 months after the last vaccination. A fraction of these effectors from three of three patients tested specifically recognized the endogenously presented E75 by indicator tumor cells. A preliminary estimation of the comparative size of the E75-specific tumor-lytic effectors performed in two patients indicated that these effectors represented 25% of the tumor-lytic effectors. This proportion is within the range described for CTL induced by foreign peptides which recognize endogenous antigen (**7,8**).

These results suggest that E75+GM-CSF delivered at multiple sites is immunogenic either for *de novo* activation of E75-specific tumor-lytic CTL, or stimulate activated/memory cells present in the patients below the levels of detection of currently used methodology. This possibility is supported by reports that peptides E75 and GP2 could activate Ag-specific tumor-lytic CTL from TIL/TAL (**13, 25-27**). Alternatively, the optimization of conditions for *in vitro* immunologic monitoring using DC, IL-12, αCTLA4 and cold-target inhibition of lysis may have increased the sensitivity of detection of these effectors.

A third possibility may be related to the use of GM-CSF at higher doses than in a recent study (**12**) and the multiple vaccinations at multiple sites. Knutson, et. al. recently reported that HER-2 peptide:369-384 mixed with two other HER-2 peptides plus GM-CSF at one half the concentration used here induced E75-specific and C85-specific CTL (**12**). PBMC from one

patient and a CTL clone from another patient after 2-3 IVS with Ag lysed targets transfected with HER-2 and HLA-A2 better than nontransfected targets. Similar results (two of two patients tested) were reported when DC-E75 were used as vaccine after three IVS (**11**). In an earlier study, two of three patients vaccinated with the same amount of E75 as in this study, plus IFA, after several IVS with E75 also increased their IFN-γ secretion but not their lytic activity to HLA-$A2^+$ HER-2^+ targets compared with their HLA-$A2^-$ HER-2^- counterparts (**10**). This suggested that E75 in the vaccine could induce partial activation of specific CTL in the vaccine.

The method for DTH measurements in this study differed significantly from that of Disis et al (28) in the following respects: (1) 50 μg of E75 was used compared to 100μg of a mixture of peptides, (2) patients were skin tested one month following four weekly injections instead of six months after six monthly injections and (3) all of the patients in our study had been heavily treated and had stage IV disease. These differences may explain the lower frequency of DTH responses >10 mm in this study (2/8 = 25%) versus 7/14 (50%) in (28). E75 (369-377) appeared to be the strongest inducer of DTH responses since DTH responses > 10 mm to longer peptides (688-703), (971-984) from other HER2 sites were less frequently observed (21.4% and 14.2%, respectively) than to E75 in this study. (**28**) The majority of patients (4 of 5) with positive DTH of 5 mm or greater proliferated to E75 either in the pre-vaccine, or post-vaccine or both. Proliferative responses to E75 post-vaccination were not significantly higher than pretreatment values. The fact that S.I. decreased post vaccination in three of four DTH^+ patients suggests that patients were presensitized to E75 prior to study, and that E75 alone provided too weak a stimulus in heavily treated, stage IV patients. There are several possible explanations for differences between DTH responses in this study and other reports: (*a*) this study involved one 9-mer peptide as immunogen, rather than mixtures of three 13-16 mer peptides (**12**); hence vaccination with E75 which is an epitope recognized by CD8+ cells on HLA-A2 alone might be less effective to induce DTH than the cumulative effects of three 13-16 were peptides, (*b*) the contribution of $CD4^+$ cells to DTH could not be excluded in Disis's study. There was a trend ($p=0.06$) for patients with positive DTH, proliferation, or both to have longer TTP. This finding must be confirmed in a larger study, preferably a randomized trial.

Collectively these studies indicated that a single peptide, E75, corresponding to a single CTL epitope together with GM-CSF could induce long-lived epitope-specific CTL that recognized the corresponding epitope on tumor cells. Specific conclusions and implications of this approach for vaccines applicable to cancer are summarized below.

The role of antigen: Vaccination with E75 induced Ag-specific tumor-lytic CTL in three of three patents tested. This was confirmed in three independent systems of testing: (a) recall responses by DC plus cytokines, (b) recall response by one stimulation with Ag *in vitro*, and (c) recall responses by three stimulations with Ag *in vitro*. While responses in the third system may be considered a result of IVS, responses in the first two systems support the hypothesis that E75-specific tumor lytic CTL were induced *in vivo* by the vaccine.

Vaccination with E75 induced E75 peptide-specific CTL in 6 of eight patients tested. An important observation from these studies was that only 4 of these eight patients responded by higher E75-specific CTL activity to IVS with E75. In two patients stimulation with E75 decreased the responses, while in two others E75 failed to induce CTL responses. This suggests

that wild-type tumor Ag vaccines may be immunogenic only in a fraction of individuals (50% in this case, or lower). For other individuals, modified tumor Ag should be used directed by immunologic monitoring. For example, it is possible that patients such as No. 6 and 9 will benefit from boosting with "attenuated" or "survival inducing immunogens" (**29**) while patients such as No. 12 and 13 from boosting with enhancer agonists, as observed with patient 13 (**24**).

The role of IL-12: Vaccination with E75-required IL-12 as costimulator for detection of IFN-γ producing cells within 24h. PBMC from 9 patients were unable to produce IFNγ following one in vitro stimulation with E75 as measured by the ELISPOT (5 pts) and/or in supernatants using ELISa (6 pts). IL-12 also enhanced our ability to detect CTL responses *in vitro*. Since E75 appeared to be a weak inducer of IL-12 and/or the frequency of E75-specific cells may be low, the use of IL-12, or of pro-inflammatory agents (CpG) during use of the *in vivo*, priming with vaccine may deserve further consideration. Again, the *ex vivo* IFN-γ and CTL responses need to be monitored to adjust the IL-12 dosage to levels where IL-12 by itself does not induce IFN-γ.

The role of CTLA-4: αCTLA-4 antibody was tested in three patients for activation of CTL and IFN-γ responses. In one patient αCTLA-4 enhanced E75-specific CTL activity above the levels observed with E75+IL-12, in the other it decreased the levels of E75-specific lysis compared with E75+IL-12. In a third patient, its CTL potentiating activity was borderline. Although this study was limited, the results suggest that the use of αCTLA-4 in immunologic monitoring may be needed to identify the presence of tolerized effectors, characterize their functional status and ability to respond to E75 by mitosis or apoptosis. Based on these determinations, αCTLA-4 may be a significant component of tumor vaccines aiming to induce tumor-specific CTL alone or together with IL-12, but its utility should be determined on a per case (or patient status) basis, particularly in patients with advanced disease who received cytotoxic treatment.

In conclusion, vaccinations with peptide E75 + GM-CSF were well tolerated by patients with advanced disease. Although E75-specific CTL could be generated in select patients, their frequencies were low requiring multiple *in vitro* stimulations with DC's with or without IL-12 and/or αCTLA-4 antibody. These findings argue for the use of IL-12 and /or αCTLA-4 (30), or consensus or specific Class II restricted helper peptides (**25**) combined with E75 to generate sufficient Th1-inducing cellular immunity, and should be considered in designing other peptide vaccine trials. Newer assays such as ELISPOT and intracellular cytokine measurements may increase the sensitivity of detection of CTL precursors, although they cannot discriminate which precursors have lytic activity. E75 or agonist peptides (**24, 26**) with higher affinity for the TCR should also be tested in cancer patients in the adjuvant or tumor-free setting where there is less likelihood for tumor immunsuppression to occur.

ADKNOWLEDGEMENTS

We thank Mrs. Samantha Caldwell and Ms. Sherry Wunsch for their outstanding editorial assistance.